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Syntheses of 1,2,3-triazolyl salicylamides with inhibitory activity on lipopolysaccharide-induced nitric oxide production

Jieun Yoon [a], Lan Cho [b], Sang Kook Lee [b,*], Jae-Sang Ryu [a,*]

[a] Center for Cell Signaling and Drug Discovery Research, College of Pharmacy and Division of Life and Pharmaceutical Sciences, Ewha Womans University, 11-1 Daehyun-Dong, Seodaemun-Gu, Seoul 120-750, Republic of Korea
[b] College of Pharmacy, Seoul National University, Seoul 151-742, Republic of Korea

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ABSTRACT

A 28-membered 1,2,3-triazolyl salicylamide library was synthesized via a Cu(I)-catalyzed azide-alkyne 1,3-dipolar cycloaddition and evaluated for their abilities to inhibit NO production in LPS-activated RAW264.7 macrophage cells. Among 28 analogues, **29g** showed a significant inhibitory activity (IC_{50} = 12.8 μM). The inhibitory effects of **29g** on LPS-mediated NO production in macrophage cells appeared to be associated with the suppression of iNOS expression.

Nitric oxide (NO) is an important signaling molecule that is involved in a wide range of pathophysiological responses such as vasodilation, nonspecific host defense, ischemia reperfusion injury, chronic inflammation and carcinogenesis. In mammals, NO is produced from the enzymatic oxidation of L-arginine by three distinct isoforms of nitric oxide synthase (NOS): neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). nNOS is located in both the central and peripheral nervous systems, and controls the production of NO, which acts as a neurotransmitter.[1] eNOS is found primarily in vascular endothelium, and regulates blood pressure and the vascular tone.[2] iNOS is ubiquitous, Ca^{2+}/calmodulin-independent, and implicated in the pathogenesis of numerous diseases. Both nNOS and eNOS are constitutively expressed, and produce NO at a low level. However, iNOS is frequently overexpressed by pro-inflammatory and/or carcinogenic stimuli such as interleukin-1β, tumor necrosis factor-α and lipopolysaccharide (LPS) in macrophages, endothelial cells and smooth muscle cells. Once expressed at high levels, iNOS is essentially unregulated, and can produce NO at cytotoxic levels, resulting in local tissue damage and many inflammatory diseases including rheumatoid arthritis, osteoarthritis, inflammatory bowel disease and multiple sclerosis. Thus, the control of NO production through iNOS inhibition is very important in the treatment of numerous disease mediated by the overproduction of NO.

The earliest inhibitors of NOS were direct analogs of the natural substrate L-arginine such as *N*-monomethyl arginine (L-NMMA, **1**),[3] *N*-nitroarginine (L-NA, **2**),[4] *N*-nitroarginine methyl ester (L-NAME, **3**), and *N*-iminoethyllysine (L-NIL, **4**)[5,6] as shown in Figure 1. Unfortunately, most of these inhibitors exhibited moderate potency and poor selectivity against NOS isoforms, which limited their application in vivo. Only GW274150 (**5**)[7] is currently under Phase II clinical evaluation for the treatment of rheumatoid arthritis and migraine. Recently, various non-peptidic small molecule inhibitors including indazole, isothioureas, amidine, 2-aminopyridines, imidazopyridine, pyrazoles and pyrroles have emerged as NOS inhibitors.[8] Such small molecules are structurally distinct from L-arginine and can thus avoid interference with physiological processes that depend on arginine transport and metabolism. In particular, AR-C102222 (**6**), one of the competitive inhibitors, showed good selectivity for iNOS and good efficacy in a rat adjuvant-induced arthritis model.[9] More recently, an imidazole-based inhibitor (BBS-4, **7**)[10] and a thiazole-based inhibitor (KLYP956, **8**)[11] were reported to be iNOS dimerization inhibitors. Mechanistically, these inhibitors disrupt the formation of the active iNOS dimer by direct coordination of the imidazole or the thiazole to the heme iron in the active site of the enzyme.[12] Although progress has been made in the development of iNOS inhibitors, there is still a need for potent and selective inhibitors that would be more drug-like and suitable for clinical use. In our ongoing efforts to develop potent, selective, and orally available non-peptidic iNOS inhibitors, we herein describe the synthesis and biological evaluation of a

* Corresponding authors. Tel.: +82 2 3277 3008; fax: +82 2 3277 2851 (J.-S.R.); tel.: +82 2 880 2475; fax: +82 2 762 8322 (S.K.L).
E-mail addresses: sklee61@snu.ac.kr (S.K. Lee), ryuj@ewha.ac.kr (J.-S. Ryu).

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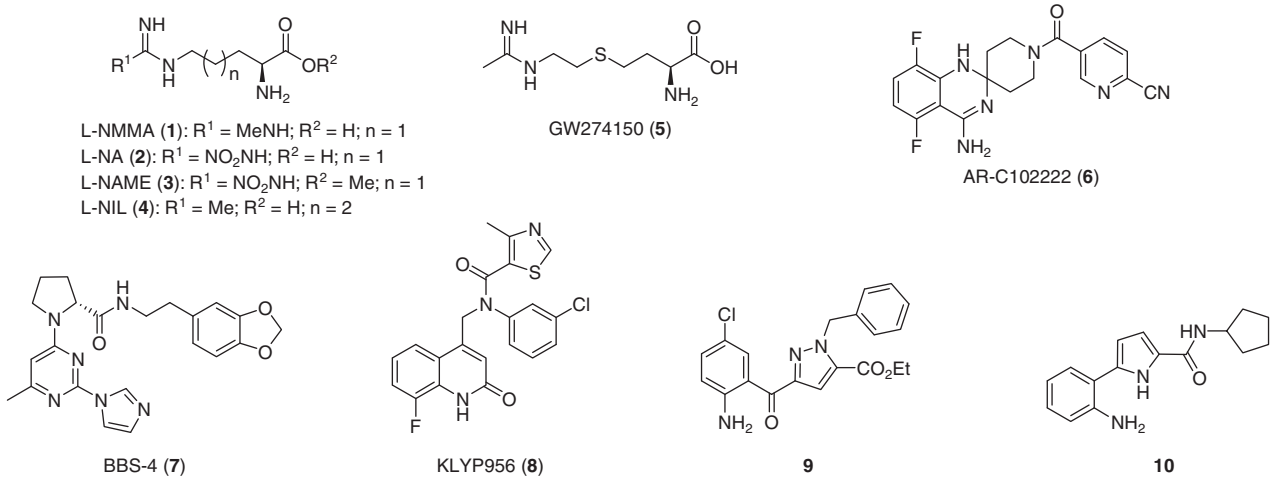
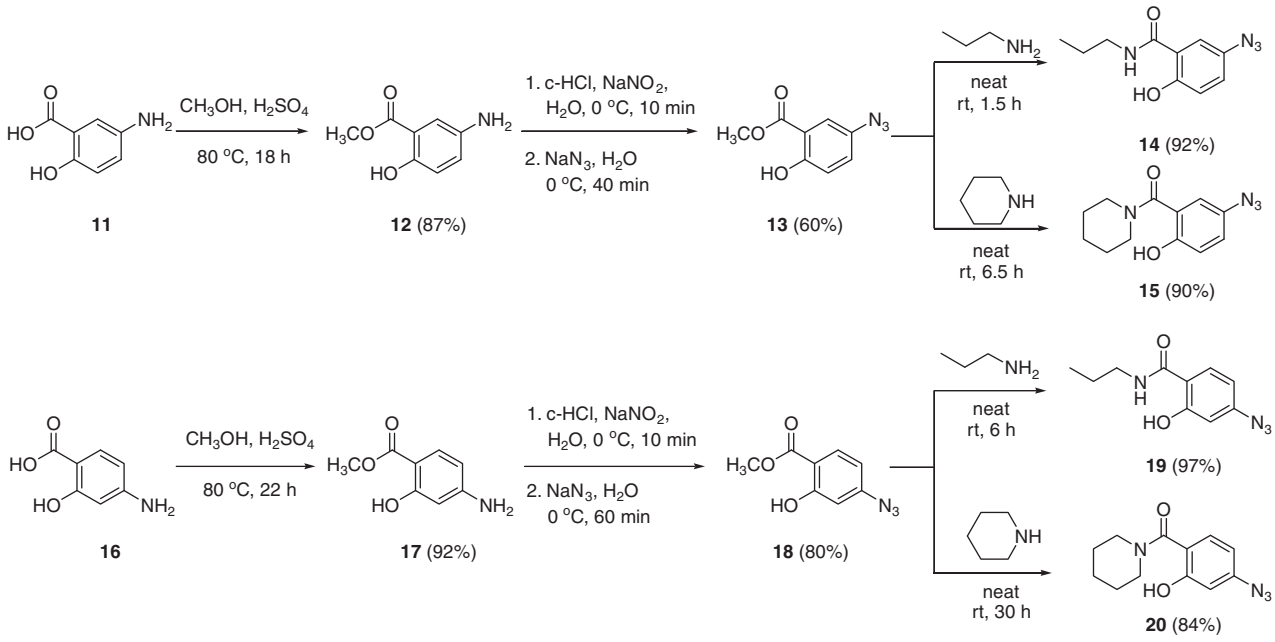


Figure 1. Structures of NOS inhibitors.



Scheme 1. Synthesis of azide building blocks.

1,2,3-triazolyl salicylamide library as a novel class of iNOS inhibitors.

Salicylamide is a non-prescription drug with anti-inflammatory, analgesic and antipyretic properties. Furthermore, the salicylamide scaffold is frequently found in several anti-inflammatory drugs and molecules. Many drugs having a salicylamide scaffold or its derivatives, are also orally available.[13] Thus, a salicylamide scaffold is a good starting point for new anti-inflammatory drug development. In addition, using a 1,2,3-triazole scaffold in iNOS inhibitor development would offer several advantages: (i) a 1,2,3-triazole can be the bioisostere of the amide bond or the heteroaromatic ring, which is observed in known inhibitors **6–10**, (ii) small molecules containing a 1,2,3-triazole moiety exhibit a wide range of biological activity, (iii) substituents can be varied through the use of different azides or alkynes, (iv) 1,2,3-triazoles can be easily assembled by click chemistry.

Click chemistry encompasses a group of powerful linking reactions that are simple to perform, have high yields, require no or minimal purification, and are versatile in joining diverse structures without the prerequisite of protection steps. In particular, the Cu(I)-catalyzed azide-alkyne 1,3-dipolar cycloaddition (CuAAC),[14] a representative process in click chemistry, provides a straightfor-

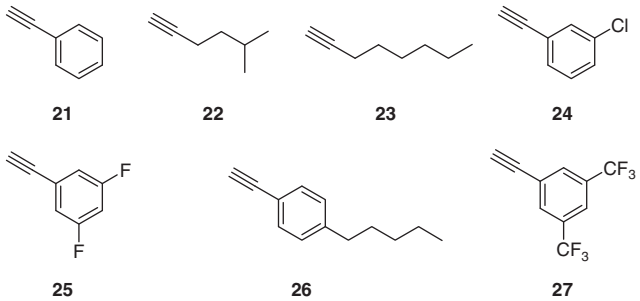
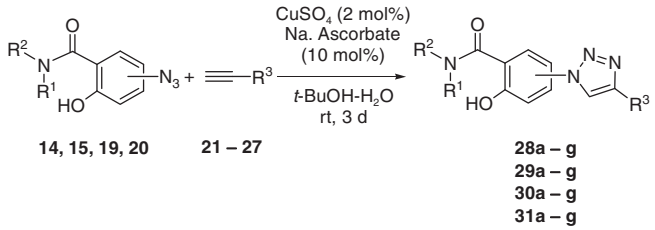


Figure 2. Structure of alkyne building blocks.

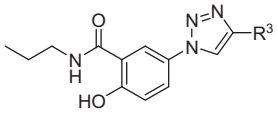


Scheme 2. Click assembly of azides and alkynes.

ward route to 1,2,3-triazoles. To access diverse 1,2,3-triazole structures, we used CuAAC. Salicylamide-based azides were prepared and coupled with various alkynes. The preparation of azide precursors **14–15** began from 5-aminosalicylic acid (**11**), which, upon treatment with methanol and H_2SO_4, was converted to the corresponding methyl ester **12** in good yield (87%, Scheme 1). Installation of an azido group in the aromatic ring was accomplished by diazotization of the amino group and treatment of the resulting diazonium intermediate with sodium azide afforded **13** in 60% overall yield. Following aminolysis with *n*-propylamine and piperidine yielded azidosalicylamides **14** and **15**, respectively. Two other compounds, the 4-azidosalicylamides **19** and **20** were synthesized in an analogous manner. In our previous study, salicylamides that obtained from arylamines and salicylic acid showed a poor aqueous solubility. Thus, in this study, only alkylamines were used to achieve better physical properties.

Alkynes **21–27** were obtained from commercial sources (Fig. 2). Synthesis of the triazole library was performed in the presence of 2 mol % of $CuSO_4$ and 10 mol % of sodium ascorbate in 28 conical tubes (Scheme 2). After three days, the resulting insoluble triazoles were precipitated by centrifugation, and the supernatant was removed by decantation. The solid residue was then washed several times with H_2O and ether to remove residual reagents. Where a solid was not formed during the reaction, solvent was removed by GeneVac™. Then, the residue was washed in the same manner. All synthesized compounds were submitted to HPLC and ^1H NMR analysis to verify their purity and authenticity. Of 28 compounds

Table 1
iNOS inhibition of 1,2,3-triazolyl salicylamides synthesized by 'click chemistry'



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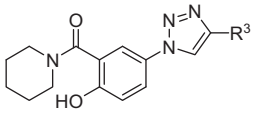

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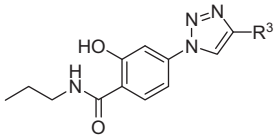

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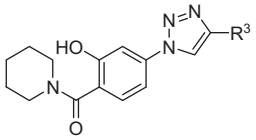

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Compound	R^3	% inhibition[a]	IC_{50} (μM)	Compound	R^3	% inhibition[a]	IC_{50} (μM)
28a	Phenyl	28.6	>20	**30a**	Phenyl	1.1	>20
28b	Isopentyl	22.6	>20	**30b**	Isopentyl	7.3	>20
28c	*n*-Hexyl	57.9	19.7	**30c**	*n*-Hexyl	11	>20
28d	*m*-Chlorophenyl	33.8	>20	**30d**	*m*-Chlorophenyl	0	>20
28e	3,5-Difluorophenyl	0	>20	**30e**	3,5-Difluorophenyl	0	>20
28f	4-*n*-Pentylphenyl	0	>20	**30f**	4-*n*-Pentylphenyl	0	>20
28g	3,5-Bis(trifluoromethyl)phenyl	1.8	>20	**30g**	3,5-Bis(trifluoromethyl)phenyl	0	>20
29a	Phenyl	11.8	>20	**31a**	Phenyl	0	>20
29b	Isopentyl	0.9	>20	**31b**	Isopentyl	1.9	>20
29c	*n*-Hexyl	4.2	>20	**31c**	*n*-Hexyl	8.3	>20
29d	*m*-Chlorophenyl	35.4	>20	**31d**	*m*-Chlorophenyl	0	>20
29e	3,5-Difluorophenyl	9.1	>20	**31e**	3,5-Difluorophenyl	0	>20
29f	4-*n*-Pentylphenyl	18.4	>20	**31f**	4-*n*-Pentylphenyl	0	>20
29g	3,5-Bis(trifluoromethyl)phenyl	94.1	12.8	**31g**	3,5-Bis(trifluoromethyl)phenyl	0	>20

[a] Inhibition at 20 μM.

synthesized, 18 were obtained in very high purity (>95%), while the others were obtained as a substrate-product mixtures, with their purities estimated to be lower than 90%. The latter compounds were resynthesized in the presence of 20 mol % of $CuSO_4$, which led to synthesis of batches with high purity.

The 28 triazole compounds were next evaluated for their ability to inhibit LPS-activated NO production by RAW264.7 macrophages. RAW 264.7 cells (5×10^5 cells/mL) were incubated in 24-well plates for 24 h. Then, LPS (1 μg/mL) and the triazole compounds were added and incubated for another 20 h. As a measure of NO produced, the accumulation of nitrite, a stable metabolite of NO in the culture supernatant was assessed using the Griess reaction[15] according to a previously documented procedure.[16] iNOS inhibition of the tested triazole compounds was expressed as % inhibition at 20 μM of inhibitor as shown in Table 1.[17] Most of the 5-(1*H*-1,2,3-triazol-1-yl)-salicylamides (**28a–d**, **28g**, **29a**, **29d–g**) inhibited LPS-induced NO production more effectively than 4-(1*H*-1,2,3-triazol-1-yl)-salicylamides (**30a–d**, **30g**, **31a**, **31d–g**). In particular, **28c** and **29g**[18] were the most potent; they exhibited 58% and 94% inhibition, respectively. Their IC_{50} values are listed in Table 1 (**28c**, 19.7 μM; **29g**, 12.8 μM). **29g** suppressed LPS-induced NO production in a dose-dependent manner (Fig. 3A). Under the same assay condition, the IC_{50} for L-NMMA (a positive control, non-selective inhibitor of NOS) was 19.9 μM. To further ensure that **29g** did not interfere with the survival of the macrophages, Raw 264.7 cells were treated with **29g** for 24 h. Cell viability was then determined by MTT assay; it was not cytotoxic at the concentrations up to15 μM (Fig. 3B).

To clarify the possible mechanism of action involved in the effects of **29g**, we examined the suppressive effects of **29g** on iNOS protein and iNOS mRNA expression in Raw 264.7 cells that were treated with LPS (1 μg/mL) in the absence or presence of various concentrations of **29g**. As illustrated in Figure 4A, treatment with

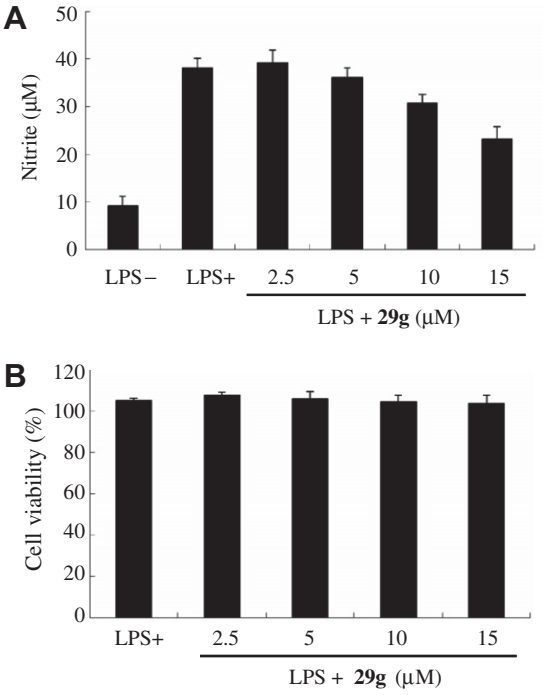

Figure 3. Effect of **29g** on LPS-induced NO production. (A) RAW 264.7 cells (5×10^5 cells/mL) were incubated in 24 well plates for 24 h, and then treated with LPS (1 μg/mL) in the presence or absence of 29g. After 20 h, supernatants were collected, and nitrite accumulation was measured by the Griess reaction. Nitrite concentrations were determined by comparison with a sodium nitrite standard curve. (B) Cell viability was measured by MTT assays as described in Supplementary data.

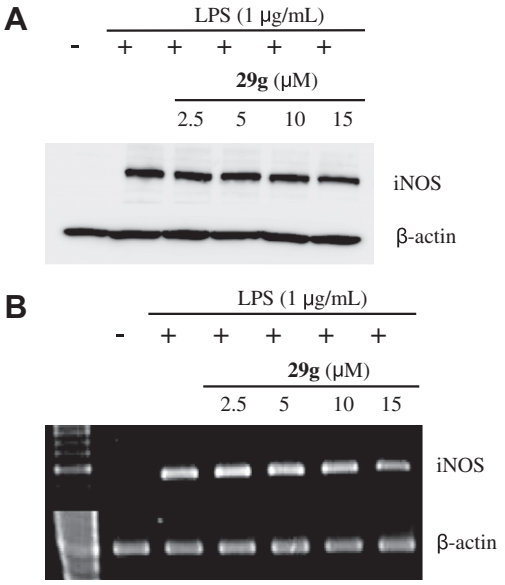

Figure 4. Modulation of iNOS protein (A) and mRNA (B) expression by **29g** in LPS-stimulated macrophages. Cells were stimulated with LPS (1 μg/mL) with or without various concentrations of **29g** for 4 h (mRNA) and 16 h (protein). Total RNA and protein were isolated and further analyzed by RT-PCR and western blots, respectively, as described in Supplementary data.

LPS (1 μg/mL) for 16 h drastically increased the expression of iNOS protein; co-treatment with **29g** suppressed the expression of iNOS protein in a concentration-dependent manner. A further study revealed that **29g** significantly inhibits, in a concentration-dependent manner, the LPS-induced increase of iNOS mRNA levels. In particular, at a concentration of 15 μM, expression of iNOS protein and RNA was significantly inhibited by **29g**. These data suggest that inhibition of NO production by **29g** is correlated with suppression of iNOS gene and iNOS protein expression.

In conclusion, a 28-membered triazole library was synthesized using a 'click chemistry' strategy. The minimum work-up using centrifugation and decantation afforded pure compounds that were suitable for the biological evaluation against iNOS. Several of the synthesized compounds inhibited LPS-activated NO production by RAW264.7 macrophages. The inhibitory effects of **29g** were associated with the suppression of iNOS expression. We believe that this compound is a good candidate for further design of non-peptidic iNOS inhibitors.

Acknowledgements

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.02.034.

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17. Nitrite assay. For measuring nitrite accumulation in the media, RAW 264.7 cells (5×10^5 cells/mL) were incubated in 24-well plates for 24 h, and then LPS (1 μg/mL) and test samples were added and incubated for 20 h. Samples were dissolved in DMSO, and final DMSO concentration in cell culture medium did not exceed 0.1%. As a parameter of NO synthesis nitrite concentration in the supernatants of culture was assessed using the Griess reaction. Briefly, 100 μl of cell culture supernatants were collected and combined with 180 μl of Griess reagent (1% sulfanilamide in 5% H_3PO_4 and 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride solution) in a 96-well plate, and then absorbance was measured at 540 nm. Nitrite concentrations were determined by comparison with a sodium nitrite standard curve. Experiments were performed at least three times with triplicate tests. In these assays, 100% activity was defined as the difference between NO production in the absence and presence of LPS for 20 h.% of Inhibition was expressed as [1 − (NO level of sample/NO level of vehicle-treated control)] × 100. IC_{50} value was calculated using nonlinear regression analyses (percent inhibition versus concentration).
18. Yield: 99%. TLC: R_f 0.24 (1:1 hexane/EtOAc). ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.48 (br s, 1H), 9.59 (s, 1H), 8.57 (s, 2H), 8.12 (s, 1H), 7.82 (dd, 1H, J = 8.8 Hz, 2.8 Hz), 7.66 (d, 1H, J = 2.8 Hz), 7.09 (d, 1H, J = 8.8 Hz), 3.60–3.22 (m, 4H), 1.65–1.60 (m, 2H), 1.58–1.50 (m, 4H). ^{1}H NMR (400 MHz, benzene-d_6): δ 11.04 (br s, 1H), 8.27 (s, 2H), 7.72 (s, 1H), 7.70 (d, 1H, J = 2.4 Hz), 7.03 (d, 1H, J = 8.8 Hz), 6.93 (s, 1H), 6.83 (dd, 1H, J = 8.8 Hz, 2.4 Hz), 3.25–3.20 (m, 4H), 1.11–1.05 (m, 6H). ^{13}C NMR (100 MHz, benzene-d_6): δ 169.5, 160.7, 145.4, 133.4, 132.5 (q, $J_{C\text{-}F}$ = 33.6 Hz), 127.0, 125.8 (m), 124.3, 123.8 (q, $J_{C\text{-}F}$ = 270.9 Hz), 121.6 (m), 121.5, 119.1, 118.6, 118.5, 46.7, 25.9, 24.2. LRMS (FAB) m/z (rel int): (pos) 485 ($[M+H]^+$, 100). HRMS m/z calcd $C_{22}H_{19}F_6N_4O_2$ 485.1412; found 485.1420.