ACS Medicinal Chemistry Letters

Letter

Riluzole-triazole hybrids as novel chemical probes for neuroprotection in Amyotrophic Lateral Sclerosis.

Joseph B. Sweeney, Marcus Rattray, Victoria Pugh, and Lucy A. Powell

ACS Med. Chem. Lett., **Just Accepted Manuscript** • DOI: 10.1021/acsmedchemlett.8b00103 • Publication Date (Web): 30 May 2018

Just Accepted

ACS Publications

is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Riluzole-triazole hybrids as novel chemical probes for neuroprotection in Amyotrophic Lateral Sclerosis.

Joseph B. Sweeney,*[†] Marcus Rattray,*[¶] Victoria Pugh,[¶,‡] and Lucy A. Powell[#]
[†]*Department of Chemistry, Lancaster University, Lancaster, LA1 4YB UK;*
[¶]*School of Pharmacy and Medical Sciences, University of Bradford, Bradford BD7 1DP, UK;*
[‡]*School of Chemistry, Food & Nutritional Sciences and Pharmacy, University of Reading, Reading, Berkshire RG6 6AP, UK;*
[#]*Department of Chemical Sciences, University of Huddersfield, Huddersfield HD1 3DH UK.*
Corresponding Authors: j.sweeney1@lancaster.ac.uk; m.rattray@bradford.ac.uk
KEYWORDS Amyotrophic lateral sclerosis, motor neuron disease, riluzole, primary cortical neurons.

ABSTRACT. Despite intense attention from biomedical and chemical researchers, there are few approved treatments for amyotrophic lateral sclerosis (ALS), with only riluzole (Rilutek®) and edaravone (Radicava®) currently available to patients. Moreover, the mechanistic basis of the activity of these drugs is currently not well-defined, limiting the ability to design new medicines for ALS. This manuscript describes the synthesis of triazole-containing riluzole analogues, and their testing in a novel neuroprotective assay. Seven compounds were identified as having neuroprotective activity, with two compounds having similar activity to riluzole.

Introduction

Amyotrophic lateral sclerosis[1] ('ALS', also known as motor neuron disease, or Lou Gehrig's Disease) involves a progressive loss of motor neurons in the central nervous system that controlling motor functions, usually resulting in death within 3-5 years after diagnosis. Though a rare condition (lifetime incidence ca. 1 in 400[2]), the disease places enormous social[3] and financial burdens on both patients[4] and healthcare programs. The condition is incurable, with riluzole[5] (**1**, Rilutek®) and edaravone[6] (**2**, Radicava®) the only drugs currently approved for treatment: both of these drugs provide short-term palliation, typically extending life by 2-3 months. In the case of familial ALS (accounting for 5-10% of cases) there is a genetic mutation which is causative,[7] but there is currently no consensus on the mode of action of the approved drugs, nor agreement on the key cellular targets for new drug-like neuroprotective molecules; thus, there is both scientific and practical value in the design and production of novel chemical matter delivering either mechanistic information or therapeutic activity for ALS.[6, 8] In addition to the two approved drug substances, there is currently a range of small molecule drug candidates[9] (including peptides,[10] saturated[11] and aromatic[12] heterocycles) being studied in trials; however, there is still a relative paucity of entirely novel chemical matter for application in ALS therapy.

Riluzole-like heterocyclic small molecule frameworks are also known to demonstrate potential neuroprotective properties; thus, pifithrin-α (**3**),[13] which contains a partially saturated bicyclic thiazolyl core (rather than the benzothiazolyl scaffold seen in riluzole), and an aromatic substituent placed at the end of a C_2-tether attached at the N_1 position. A similar substituent pattern is seen in riluzole analogues **4a** and **4b** (Figure 1)[5] with the presence of a functionalized ethyl sidechain endowing neuroprotection activity at similar levels to riluzole in rodent models.[5] As a novel chemical target with potential for neuroprotection, and bearing in mind the pyrazinone core of edaravone, we have synthesised previously unreported triazoles **5** as new chemical matter with structural resemblance to riluzole and related molecules.

The synthetic strategy to these compounds revolved around previously unknown lynchpin azide **6**, which would undergo copper(I)-catalysed cycloaddition with a range of alkynes to give **5** (Scheme 1). In addition to the triazole functionality being a known pharmacophoric motif in marketed drug substances (such as tazobactam and

cefatrizine), we anticipated that the modularity of the Click process also would allow for incorporation of other functionality (diazirine, dye labels etc.), thereby facilitating future mechanistic studies.

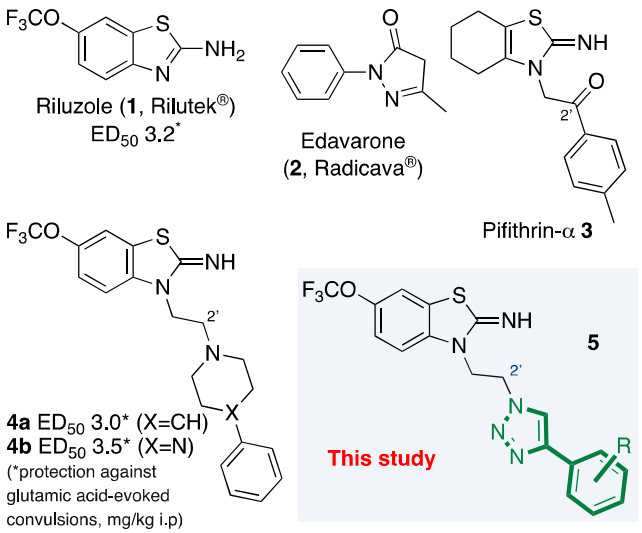
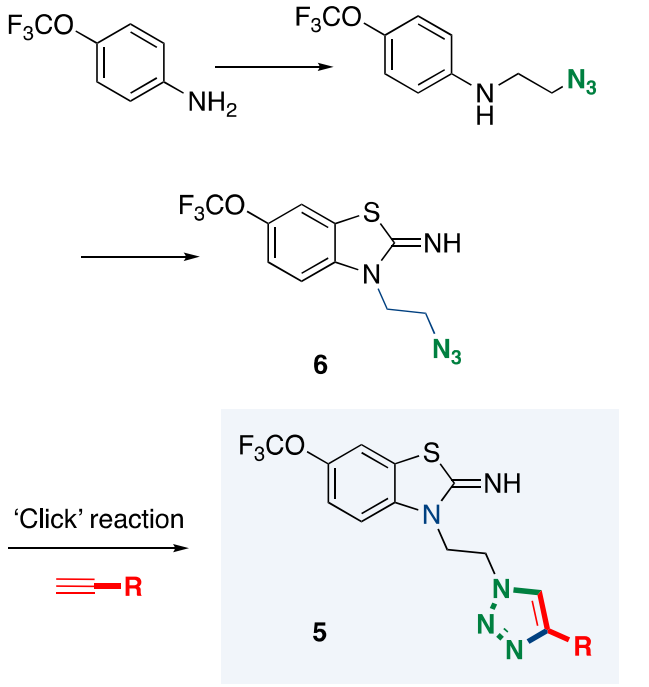


Figure 1. ALS drugs (**1** and **2**), neuroprotective small molecules (**1** and **2**), and project target (**5**)

A major challenge in the design of new therapies for ALS is the fact that the binding site of riluzole remains elusive, precluding definitive conclusions to be reached about the mode of action of approved drug substances and novel chemical matter alike. Previous studies using radioligand binding failed to demonstrate any interactions between riluzole and known ligand binding sites on the kainate and NMDA receptors, or the $GABA_A$ (γ-aminobutyric acid) or glycine receptors.[14, 15, 16] *in silico* docking analysis highlighted the $Na_v1.6$ channel[17]. Since conclusive data to confirm the mechanism of action and binding modes of riluzole remain elusive, limiting the further development of riluzole in the therapeutic pipeline, we envisage tagged chemical probes will facilitate subsequent mechanistic studies once activity has been established.

A complication of the methods used to study the design and development of chemotherapeutics is the fact that the experimental preparations used to determine the mechanism of action of existing and new drugs and drug-like substances vary considerably, with many species, cell types protocol in use. Significantly, many of the cell lines used in the study of ALS are different to the cell types primarily affected in the disease. This presents a challenge in direct comparison of data, and makes it difficult both to identify new drugs and to delineate mechanistic rationales. We describe here the synthesis of a novel library of hybrid small molecules containing features of both riluzole and edaravone, and the screening of this new chemical matter in novel neuroprotective assays.



Scheme 1. Click chemical strategy for preparation of triazole library **5**.

Experimental details

In order to screen riluzole derivatives, and because of the paucity of robust in vitro assays in this field it was important to first determine the most suitable cell type, stimulus of cell death and measure of cell death to measure neuroprotection. Mouse primary cortical neurons and primary motor neurons were cultured using previously described methods[18]. For screens, neuroprotection assays based on MTT turnover, cell counting, caspase activation were discarded as riluzole or derivatives were not protective in these assays of cell death (results not shown). Since dendritic damage and loss is a cardinal feature of the slow degeneration found in motor neuron disease states we developed morphometric analyses to measure neuronal complexity following disease-

relevant stimuli. From a number of challenges tested, the AMPA receptor agonist, kainate was selected to elicit cell damage without rapid cell death. For primary cortical neurons an antibody against microtubule-associated protein, MAP2 was used in Western blotting experiments and also to stain neuronal dendritic processes. Volocity software was used for perimeter analysis of cell profiles stained with MAP2 and each experimental condition was normalized to the kainate only condition.

Chemical matter. The target triazole library was accessed in short order on hundred-milligram scale according to the synthetic strategy shown in Scheme 1. Thus, Click reaction of the previously unreported lynchpin azide **6** with a range of alkynes, directly delivered previously unreported triazoles **5a**-**5aab** (Table 1). Armed with this library of novel chemical matter, we proceeded to the testing program using *in vitro* model systems.

Results

The library of riluzole derivatives **5a**-**5aab** were tested on primary cortical neurons for their ability to protect against kainate induced dendritic loss. From this assay, seven compounds were identified as having promise (Figure 2, supporting information). Test compounds **5ap**, **5ao**, **5r**, **5g**, **5aq**, **5w** and **5ak**, but not the parent, riluzole (ril), attenuated kainate-induced neurofilament loss. The remaining members of the triazole library did not prevent kainate-induced neurofilament loss (data not shown).

The seven compounds that were positive in the MAP2 assay were also screened in primary cortical neurons, using plate-based technology to assay functional properties. In healthy neurons K^+ can be used to depolarise neurons and initiate a Ca^{2+} flux through voltage gated calcium channels. This flux was monitored through incubating cells with fura-2AM for 45 mins, then washing and challenging with 10 mM KCl to induce calcium entry. Neurons pretreated with kainate exhibited a reduced calcium flux, indicating compromised cell function. Riluzole and all the riluzole derivatives were able to attenuate this effect, with **5ap** and **5g** having the greatest effect (results not shown).

We next determined protection using a secondary screen of primary mouse spinal cord motor neurons using the positive compounds from the cortical neuron screen. We first tested a range of outcome assays including cell counting and plate reading of SMI-32 fluorescence, but none provided an output that was both sensitive to disease-relevant stimuli and rescued by riluzole. We therefore employed the antibody SMI-32[19] to label neurofilaments of motor neurons, and Sholl analysis[20] to monitor neuronal complexity. Following treatments, cells were fixed and stained with SMI-32 antibody and fluorescent images of cells were captured. Ten individual cells were analysed for each condition, and the experiment was repeated three times and the data pooled. For Sholl analysis, concentric circles at each 100 μm radius from the cell centre were drawn, and the number of intersections of processes at each radius interval were measured using image analysis software. The data provided an indication of the arborization and complexity of the motor neuron processes, with reduced process complexity and length preceding cell death. Figure 3 (supporting information) shows representative images and analysis from these experiments. Riluzole showed a significant neuroprotective effect, as did two of the test compounds, **5ap** and **5ao**.

Discussion

Phenyl derivatives. Phenyl triazole **5a** was capable of preventing kainate-induced MAP2 fluorescence loss above that of the parent compound, riluzole. However triazoles in which the aryl motif was placed at increasing distances from the heterocycle unit: increasing the length of the carbon chain between the triazole and phenyl ring were not effective, with compound **5b** (placed at the terminus of a 2-carbon tether) and **5at** (with a 3-carbon tether) showing reduced neuroprotection compared with parent **5a**. Compound **5a** was not effective in the secondary screen in motor neurons.

Substituted phenyl derivatives. In compounds **5g**-**5l** the spatial arrangement of the carbon tether is changed, with the saturated motif placed on the phenyl ring as opposed to the triazolyl unit. Compound **5g**, bearing a *para*-C_2-substitutent showed significant activity against kainate-induced loss in MAP2 in cortical neurons while compounds **5h**, **5i**, **5k**, and **5l** (bearing linear *para*-carbon chains of 6, 5, 4, and 3 carbons, re-

spectively) did not significantly increase MAP2 fluorescence above that of kainate alone. Compound **5g** attenuated kainite reduction of calcium flux, but was not effective in the secondary screen in motor neurons.

Alkyl triazoles. Deletion of the phenyl ring of the scaffold and replacement with alkyl substituents (**5aw, 5ax, 5au**) did not yield compounds with neuroprotective ability as determined by the MAP2 fluorescence assay.

Hetarene derivatives. Pyridyl-substituted triazoles **5an-5ap** included the two most promising compounds. **5ap** (with a 3'-substitution pattern) and **5ao** (4'-substitution) both demonstrated neuroprotection, reducing kainate induced loss in MAP2, and also preventing motor neuron damage in vitro, with **5ap** rescuing kainate-induced reduction in calcium flux in cortical neurons.: We note both **5ao** and **5ap** are more active than 2'-pyridyl **5an** which demonstrated increased MAP2 levels but not at a high-enough activity to carry forward into the secondary screen. The 2'-Thiophenyl triazole **5aq** was protective in reducing kainite-induced MAP2 loss but, the 3'-isomer, **5ar** had no positive activity.

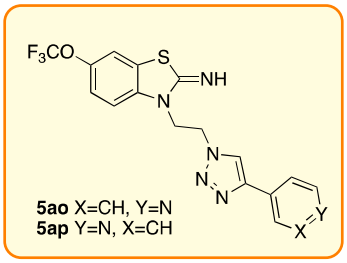
Electron-rich aryl derivatives. Compound **5r**, protected MAP2 fluorescence from kainate treatment, has the addition of an amino group at position 6. Similar derivatives include compounds **5p** and **5q** which have the amino group at position 4 and 5 respectively, but neither afforded significant neuroprotection, with neuron. Compounds **5c**, **5d** and **5e** had the inclusion of a methyl group onto the phenyl ring, at positions 4, 5 and 6 respectively, but none exhibited neuroprotection.

Electron-deficient aryl derivatives. *para*-(Bromo)phenyl triazole **5w** prevented kainate-induced MAP2 loss in the primary MAP2 screen but was ineffective on motor neurons, Its *ortho*-isomer **5x** had no effect. Chlorination was uniformly unsuccessful: compounds **5t-v** exhibited no neuroprotection activity. Mono- (compounds **5y-5aa**), di- (**5ab-5ad**) or trifluorinated (**5ae**) compounds exhibited no neuroprotection activity, while *para*-(fluoro)phenyl compound **5z** induced increased MAP2 fluorescence but not to an extent great enough to merit further screening. (Trifluoromethyl)phenyl triazoles (**5af-5ah**) did not exhibit protection. The *para*-(Carboxy)phenyl triazole **5ak**, increased, but the corresponding ester **5aj**, decreased MAP2 fluorescence compared to kainate alone, and demonstrated toxicity in its own right (results not shown).

These data offer the promise of using entirely novel chemical matter for application in ALS therapy, either as drug-like substances or chemical probes. The next phase in this research will focus on structural modification and SAR of the small molecules with riluzole-like activity (compounds **5ao** and **5ap**), and on the use of less active compounds (compounds **5w** and **5ak**) as start-points for design and implementation of chemical probes (such as 'Click' reagents).

Summary

Using a riluzole-triazole hybrid library **5a-5aab** and a novel motor neuron screen, we have identified seven new triazoles with neuroprotective activity greater than the parent, riluzole. Of this subset our data shows that the pyridyl-substituted compounds **5ao** and **5ap** are the most promising, with neuroprotective properties greater than riluzole in two independent in vitro assays on primary neurons. These represent promising chemical start-points for mechanistic studies. We are currently engaged in the design and delivery of chemical probes based on these structures, and in the application of this novel chemical matter to further studies of ALS and related conditions.



ASSOCIATED CONTENT

Supporting Information

Experimental details for preparation of compounds **5a-5aab**, including characterization data (PDF).

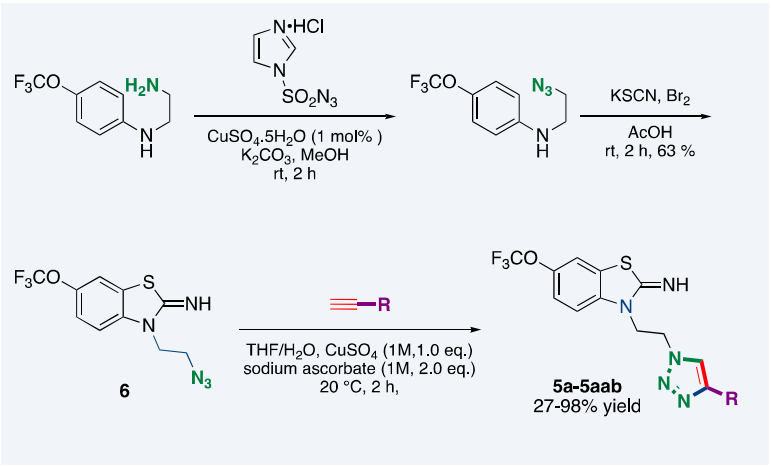
ACKNOWLEDGMENT

We thank the Amyotrophic Lateral Sclerosis Association (ALSA) for project funding, and the Motor Neurone Disease Association, the University of Reading (PhD grant to V. P.) and AstraZeneca (PhD grant to L. A. P.) for other funding.

Lay summary. Motor neuron diseases, such as ALS – Lou Gehrig's disease – are very difficult to treat: this is partly due to the lack of understanding of the way the diseases works, and also due to the lack of new medicines which are effective. This project is focused on making new drug-like molecules, hoped to be effective in providing protection to neurons similar to those damaged in ALS. The study identified two new structures which could protect neurons; these compounds are therefore promising new tools in the treatment and understanding of ALS.

References

1. Kiernan, M. C.; Vucic, S.; Cheah, B. C.; Turner, M. R.; Eisen, A.; Hardiman, O.; Burrell, J. R.; Zoing, M. C.; Amyotrophic lateral sclerosis. *Lancet* **2011**, *377*, 942–55.
2. Alonso A.; Logroscino G.; Jick S. S.; Hernán M. A. *Eur. J. Neurol*. **2009**, *16*, 745-51.
3. Gibbons C. J.; Thornton, E. W.; Ealing, J.; Shaw, P. J.; Talbot, K.; Tennant, A.; Young, C. A.. Assessing social isolation in motor neurone disease: A Rasch analysis of the MND Social Withdrawal Scale. *J Neurol Sci.* **2013**, *334*, 112–118.
4. It has been estimated that ALS patients and their families incur costs of ca. $14k p.a. as a direct result of the disease: https://www.demos.co.uk/wp-content/uploads/2017/06/DEMJ5449_Financial_impact_report_o90617_WEB-6.pdf.
5. Jimonet P.; Audiau F.; Barreau M.; Blanchard J. C.; Boireau A.; Bour Y.; Coleno M. A.; Doble A.; Doerflinger G.; Huu C. D.; Donat M. H.; Duchesne J. M.; Ganil P.; Gueremy C.; Honor E.; Just B.; Kerphirique R.; Gontier S.; Hubert P.; Laduron P. M.; Le Blevec J.; Meunier M.; Miquet J. M.; Nemecek C.; Pasquet, M.; Piot, O.; Pratt, J.; Rataud, J.; Reibaud, M.; Stutzmann, J.-M..; Mignani S.; Riluzole series. Synthesis and in vivo "antiglutamate" activity of 6-substituted-2-benzothiazolamines and 3-substituted-2- imino-benzothiazolines. *J Med Chem* **1999**, *42*, 2828-2843.
6. Kiernan, M. C.; Motor neuron disease in 2017: Progress towards therapy in motor neuron disease. *Nature Reviews Neurology* **2018**, *14*, 65–66.
7. Rosen, D. R.; Siddique, T.; Patterson, D.; Figlewicz, D. A.; Sapp, P.; Hentati, A.; Donaldson, D.; Goto, J.; O'Regan, J. P.; Deng, H.-X.; Rahmani, Z.; Krizus, A.; McKenna-Yasek, D.; Cayabyab, A.; Gaston, S. M.; Berger, R.; Tanzi, R. E.; Halperin, J. J.; Herzfeldt, B.; Van den Bergh, R.; Hung, W.-Y.; Bird, T.; Deng, G.; Mulder, D. W.; Smyth, C.; Laing, N. G.; Soriano, E.; Pericak–Vance, M. A.; Haines, J.; Rouleau, G. A.; Gusella, J. S.; Horvitz, H. R.; Brown Jr, R. H.; Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature* **1993**, 362, 59–62.
8. Özdinler, P. H.;Silverman, R. B.; Treatment of Amyotrophic Lateral Sclerosis: Lessons Learned from Many Failures. *ACS Med. Chem. Lett*. **2014**, 5, 1179–1181.
9. See: ALS Research Forum 'Drugs In Development Database': http://www.alsresearchforum.org/research-tools/drugs-in-development-database.
10. Bainan, W.; De, S. K.; Kulinich, A.; Salem, A. F.; Koeppen, J.; Wang, R.; Elisa Barile, E.; Wang, S.; Zhang, D.; Ethell, I.; Pellecchia, M. Potent and Selective EphA4 Agonists for the Treatment of ALS. *Cell Chem. Biol*. **2017**, *24*, 293–305.
11. Mavlyutov, T. A.; Guo, L. W.; Epstein, M. L.; Ruoho, A. E.; Role of the Sigma-1 receptor in Amyotrophic Lateral Sclerosis (ALS). *J Pharmacol Sci.* **2015**, *127*, 10-6.
12. See, for instance: Benatar, M.; Wuu, J.; Andersen P. M.; Atassi, N.; David W.; Cudkowicz, M.; Schoenfeld, D. Randomized, double-blind, placebo-controlled trial of arimoclomol in rapidly progressive SOD1 ALS. *Neurology* **2018**, *90*, e565-e574.
13. Zhu, X.; Yu, Q.-s; Cutler, R. G.; Culmsee, C. W.; Holloway, H. W.; Lahiri, D. K.; Mattson, M. P.; Greig, N. H.; Novel p53 Inactivators with Neuroprotective Action: Syntheses and Pharmacological Evaluation of 2-Imino-2,3,4,5,6,7-hexahydrobenzothiazole and 2-Imino-2,3,4,5,6,7-hexahydrobenzoxazole Derivatives. *J. Med. Chem.* **2002**, *45*, 5090-5097.
14. Benavides J., Camelin J. C., Mitrani N., Flamand F., Uzan A., Legrand J. J., Gueremy C., Le Fur G. 2-Amino-6-trifluoromethoxy benzothiazole, a possible antagonist of excitatory amino acid neurotransmission-II. Biochemical properties. *Neuropharmacology* **1985**, **24**, 1085-1092; Debono M. W.; Le Guern J.; Canton T.; Doble A.; Pradier L. Inhibition by riluzole of electrophysiological responses mediated by rat kainate and NMDA receptors expressed in Xenopus oocytes. *Eur. J. Pharmacol.* **1993**, *235*, 283-289; He Y.; Benz A.; Fu T.; Wang M.; Covey D. F.; Zorumski C. F.; Mennerick S. Neuroprotective agent riluzole potentiates postsynaptic GABA(A) receptor function. *Neuropharmacology* **2002**, *42*, 199-209.
15. Debono M. W.; Le Guern J.; Canton T.; Doble A.; Pradier L. Inhibition by riluzole of electrophysiological responses mediated by rat kainate and NMDA receptors expressed in Xenopus oocytes. *Eur. J. Pharmacol.* **1993**, *235*, 283-289.
16. He Y.; Benz A.; Fu T.; Wang M.; Covey D. F.; Zorumski C. F.; Mennerick S. Neuroprotective agent riluzole potentiates postsynaptic GABA(A) receptor function. *Neuropharmacology* **2002**, *42*, 199-209.
17. Sierra Bello O.; Gonzalez J.; Capani F.; Barreto G. E. *In silico* docking reveals possible Riluzole binding sites on Nav1.6 sodium channel: implications for amyotrophic lateral sclerosis therapy. *J. Theor. Biol*. **2012**, *315*, 53-63.
18. Rainey-Smith S. R.; Andersson D. A.; Williams R. J.; Rattray M. Tumour necrosis factor alpha induces rapid reduction in AMPA receptor-mediated calcium entry in motor neurones by increasing cell surface expression of the GluR2 subunit: relevance to neurodegeneration. *J Neurochem.* **2010**, *113*, 692-703
19. Tsang Y. M.; Chiong F.; Kuznetsov D.; Kasarskis E.; Geula C. Motor neurons are rich in non-phosphorylated neurofilaments: cross-species comparison and alterations in ALS. *Brain Res.* **2000**, *861*, 45-58.
20. Sholl, D.A. Dendritic organization in the neurons of the visual and motor cortices of the cat. *J. Anat.* **1953**, *87*, 387–406.



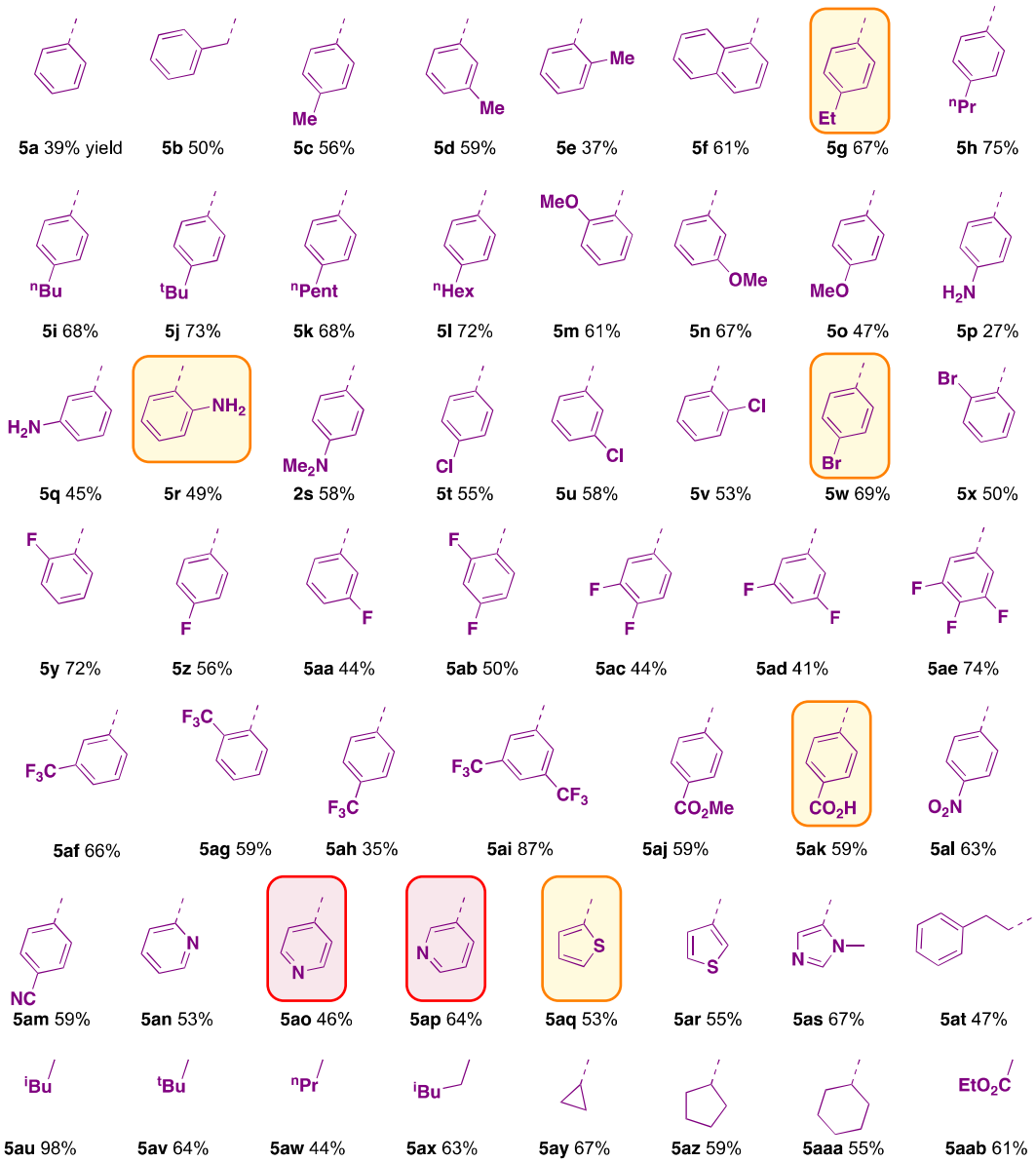
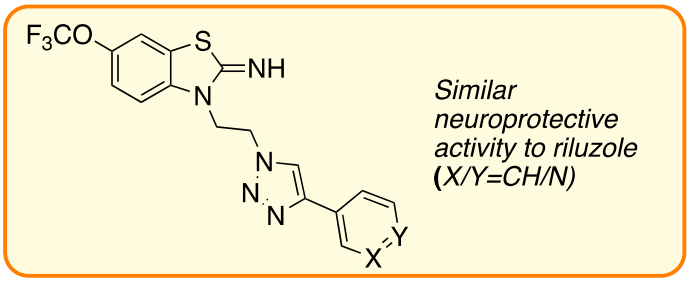


Table 1. Preparation of triazolyl library **5a-5aab** (active compounds highlighted)



Similar neuroprotective activity to riluzole (*X/Y=CH/N*)