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Short communication

Design, synthesis and biological evaluation of small-azo-dyes as potent Vesicular Glutamate Transporters inhibitors

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ABSTRACT

Vesicular Glutamate Transporters (VGLUTs) allow the loading of presynapic glutamate vesicles and thus play a critical role in glutamatergic synaptic transmission. VGLUTs have proved to be involved in several major neuropathologies and directly correlated to clinical dementia in Alzheimer and Parkinson's disease. Accordingly VGLUT represent a key biological target or biomarker for neuropathology treatment or diagnostic. Yet, despite the pivotal role of VGLUTs, their pharmacology appears quite limited. Known competitive inhibitors are restricted to some dyes as Trypan Blue (TB) and glutamate mimics. This lack of pharmacological tools has heavily hampered VGLUT investigations. Here we report a rapid access to small molecules that combine benefits of TB and dicarboxylic quinolines (DCQs). Their ability to block vesicular glutamate uptake was evaluated. Several compounds displayed low micromolar inhibitory potency when size related compounds are thirty to forty times less potent (i.e. DCQ). We then confirmed the VGLUT selectivity by measuring the effect of the series on vesicular monoamine transport and on metabotropic glutamate receptor activity. These inhibitors are synthesized in only two steps and count among the best pharmacological tools for VGLUTs studies.

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1. Introduction

Glutamate (Glu) is the major excitatory neurotransmitter in the Central Nervous System (CNS) and is involved in most, if not all, physiological functions as well as neuropathologies of the CNS [1]. To secure synaptic transmission, the concentration of Glu in the synaptic vesicles has to be finely regulated during the neurotransmitter cycle [2]. Glu released from synaptic vesicles by exocytosis binds to glutamate receptors to trigger biological responses. Released glutamate is rapidly removed from the synaptic cleft by plasma membrane transporters named EAAT1-5 (Excitatory Amino Acid Transporters) [3]. Reuptaken Glu is accumulated into synaptic vesicles by VGLUT1-3 for additional cycles of release [4,5].

In this study, we focus our attention on one step of this glutamate cycle: the vesicular uploading and accumulation by VGLUTs. Despite their key role in excitatory transmission, pharmacology of VGLUTs appears very poor. Indeed, only few chemical compounds efficiently target these transporters [3]. Accordingly development of pharmacological tools appears crucial to better understand the functional implication of VGLUT1-3 in normal and pathological conditions and to use these transporters as biomarkers of human "synaptopathologies" for diagnosis or therapeutic purposes. In spite of their recent characterization, therapeutic implication has been demonstrated in Parkinson's disease model, Alzheimer disease or epilepsy [6]. Presently, three types of VGLUT inhibitors have been identified [3] : dyes [5,7–10] with Ki in the 20 nM–10 μM range, substituted quinolines (DCQ) [11,12] with Ki in the 40–300 μM range and glutamate analogs with IC_{50} > 230 μM. Dyes display higher affinities, but a lower selectivity than quinolines

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[13]. The third type includes the glutamate-like inhibitors known to interact with glutamate receptors but these compounds show very weak affinity on VGLUT [3]. We have previously shown that potency of Rose Bengal (RB) may not be increased by chemical modulation [10]. On the other hand Trypan Blue (TB) or Evans Blue (EB) are well known universal cellular dyes thus lacking selectivity and chemical variations of these dyes are not easily accessible. DCQ derivatives remain a potential structure in terms of pharmacological purpose but Carrigan et al. have shown their limit considering their moderate affinities. Moreover these derivatives have been synthesized in very low yields (<5% for best compounds) [12,14]. In this work, we propose to increase the potency and improve the synthesis of these derivatives by replacing DCQ by the di-sulfonic naphthyl moiety present in TB inhibitor as a bioisostere. DCQs (Fig. 1) can be replaced by the newly synthesized analogs in high yields and in less synthetic steps. Comparison between the dyes' structures and DCQs, allowed the design of a hybrid azo-linked-biaryl-disulfonic- naphthyl shown in Fig. 1 and discussed below. Synthesis of such a series and VGLUT uptake evaluation are described herein. Compounds **7c**, **7e**, **4i** and **4p** are the most potent derivatives with IC_{50}'s of 1.6, 1.8, 2.1 and 2.1 μM respectively. These new VGLUT inhibitors provide a valuable contribution to the pharmacology of these transporters that is amazingly limited.

2. Results and discussion

2.1. Chemistry

Analogs of the mono-azo dyes **4** where generated by modulation of the phenyl ring substituents while keeping the same di-sulfonic naphthalene entity as deduced from observed variations of EB (IC_{50} = 87 nM), TB (IC_{50} = 50 nM) and Chicago Sky Blue (CSB) (IC_{50} = 3 μM) potencies. Indeed EB and TB display similar VGLUT inhibitory activity whereas replacing the methyl group of EB by a methoxy group in CSB decreases 34-fold the inhibitory activity [9,15]. SAR of DCQs also revealed the critical role of aromatic substitution [11,12]. We also decided to investigate the phenyl ring substituent effect in **4** by introducing electro-donating or -withdrawing groups. Since in the quinoline series, the type of bond between the phenyl and quinoline moiety has little effect on inhibitory activity [12], we selected a diazo-link for its synthetic simplicity. In order to increase solubility, we selected *p*-carboxylic acid substituted analogs and corresponding *p*-halogeno analogs to increase hydrophobicity. The major advantage of the diazo-naphtalene bioisostere is its synthetic route allowing easy access to various derivatives such as compounds **4** and **7** (Scheme 1). They may be obtained in only two steps performed in one pot: diazotation of aniline followed by electrophilic aromatic substitution of a naphthyl derivative. Due to the relative instability of diazonium salt, we prepared this intermediate in situ and used it directly, without purification. Nevertheless kinetics of diazonium formation needs to be controlled when used directly.

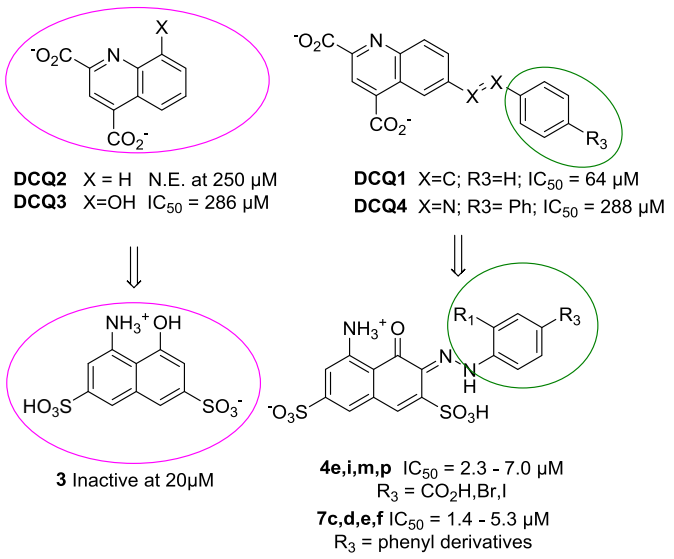
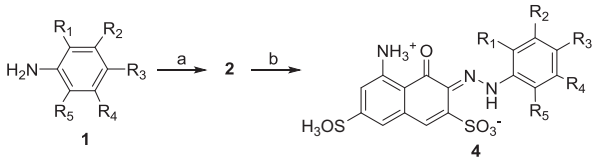


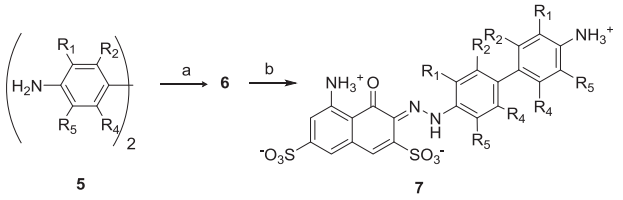
Fig. 1. Strategy used for compounds **4** and **7** design: we combined quinoline key features (i.e. hydrophobic substituent (circled in green), aromatic bicyclic negative charges (circled in pink)) and easily accessible naphthyl blue dyes counterpart (i.e. Compound **3**). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

As previously mentioned, the first step consists in the formation of a diazonium salt **2** or **6** in acidic aqueous solution. The mechanism of the reaction has been already described by several groups since 1958 [16,17] and different mechanisms postulated. To clarify the kinetics, we monitored the diazonium/aniline ratio obtained in various conditions by ^{1}H NMR. As anticipated, electronic properties of aniline substituents highly impact the diazotation kinetics. Indeed, deactivation of the aromatic ring by electron-withdrawing or sigma-attracting substituents (i.e. nitro or fluorine) led to a mixture of diazonium compounds and starting material (Supplementary Fig. 1 and Table 1). Moreover when aniline was unsubstituted or substituted by an electron-donating group, the reaction was complete. Thus, two major factors appear to directly influence reaction completion: the aniline nucleophilicity and its solubility in acidic media. Comparison between pKa and reaction completion clearly indicates that diazonium formation is limited by aniline nucleophilic attack on nitrosonium ion (assuming that amine pKa can be correlated to its nucleophilicity). But this only factor may not explain the difference between fluorine or chlorine substituted anilines diazotation. We also observed that aniline solubility is critical as illustrated when using poorly soluble 2,4-dinitro aniline. No reaction was observed in water while 70% of conversion was estimated when reaction was run in ethanol as solvent. This particular reactivity is even more spectacular when working on the mono-diazotation of benzidine derivatives **5**. As for aniline, benzidine holding withdrawing substituents (2-F or 2-Cl) reacts partially with sodium nitrite and leads to only mono-diazotized derivative. In contrast, as already observed with aniline, benzidine bearing electro-donating or sigma-donor substituents (−Me, −OMe) are more reactive and when treated with even less than 1 equivalent of sodium nitrite a mixture of di-diazonium salt, starting material and mono-diazotized analog is obtained. Taking into account these observations, diazotation reaction of electron-poor aromatic anilines has been performed in water/ethanol solvent using 1.3 equivalents of sodium nitrite. Concerning compound **7**, a preliminary study has been limited to easily accessible diazotized benzidine (electro-withdrawing substituted aniline).

The second step of the synthesis of **4** and **7**, consists in an aromatic electrophilic substitution of a poly-functionalized naphthyl moiety (Compound **3**). Again at this step, literature appears quiet unreliable. When some authors described an attack specifically on the ortho position of a phenol [18–20], others confirmed the regioselective addition in the para position of the hydroxyl substituent [21]. Based on this literature, aromatic substitution should also occur in ortho/para position of the hydroxyl substituent in alkaline conditions and in ortho position of the amino group in acidic ones (Supplementary Fig. 1). To avoid side product formation and a fastidious purification step when using compound **2** bearing both amino and hydroxyl substituent, we decided to clarify the regioselectivity of this step and thus studied first diazonium



	R_1	R_2	R_3	R_4	R_5		R_1	R_2	R_3	R_4	R_5
4a	CH_3	H	CO_2H	H	H	**4i**	CH_3	H	I	H	H
4b	H	H	CO_2H	H	H	**4j**	H	H	Br	H	H
4c	Cl	H	CO_2H	H	H	**4k**	Cl	H	I	H	H
4d	F	H	CO_2H	H	H	**4l**	F	H	I	H	H
4e	CF_3	H	CO_2H	H	H	**4m**	CF_3	H	I	H	H
4f	F	F	CO_2H	F	OH	**4n**	F	F	Br	F	OH
4g	OCH_3	H	CO_2H	H	H	**4o**	OCH_3	H	Br	H	H
4h	CH_3	H	CO_2H	H	CH_3	**4p**	CH_3	H	Br	H	CH_3



	R_1	R_2	R_4	R_5		R_1	R_2	R_4	R_5
7a	CH_3	H	H	H	**7d**	F	H	H	H
7b	H	H	H	H	**7e**	CF_3	F	F	F
7c	Cl	H	H	H	**7f**	F	F	F	F

Scheme 1. Synthesis of compounds **4** and **7**. a) $NaNO_2$, HCl/H_2O, b) Compound **3**, NaOH 10%, pH = 9.

addition in simpler structures (compounds **8**, **10**, **12**, **14**, Scheme 2) [22,23].

A representative diazonium salt (compound **2k**) was added to a range of naphthyls (**8**, **10**, **12**, **14**) at pH 9.0. Mono or di-additions was confirmed firstly by Mass spectrometry. Regioselectivity of additions were elucidated by NMR spectroscopic experiments (NOESY, HMQC…) (See Supplementary data). As expected, compound **8** gave only one substituted isomer **9** diazotized in C1 position (i.e. no other para or ortho position was accessible) in a quantitative yield after 1 h of stirring at 0–5 °C. Regioselectivity was confirmed by ^{1}H NMR and ^{13}C NMR: the doublet between H5 and H6 of naphthyl moiety confirmed that no addition occurred on the corresponding carbons. Moreover the chemical shift of C3 characteristic of a ketone function (δ (C3) >170 ppm) established that the coupling occurred in C4 position.

When diazonium **2k** was added to compound **10** in the same conditions, only single ortho substitution (product **11**) occurred and no addition in para position of the hydroxyl was detected [24]. As previously described no addition occurred in C5 and C6 positions (presence of doublets in the naphtyl moiety correlating with the ketone C3 in HMBC experiment). Cosy correlation between H6 and H8 and HSQC experiment permitted the identification of H8 and C8. Finally, a correlation between C8 and a singulet (H1) in HMBC experiment confirmed the addition on C3.

Surprisingly, when compound **12** was treated in these conditions, only the di-substituted compound **13** was obtained. At this step no mono-addition was observed with mass spectrometry and the single pic of di-coupled compounds ($[M-H]^- = 766$ in ESI-) was detected. Moreover, the doublet between H5 and H6 of naphtyl moiety confirmed that no addition occurred on the corresponding carbons. The di-addition could be either at the C3 and C1 positions or C3 and C8 or C8 and C1. Nevertheless, the NOESY experiment highlighted a correlation between a proton of the aryl substituent (H–C6″) and a proton presents on the naphthyl moiety. This ruled out an addition on the C1 and C8 position. To discriminate between an addition on C1–C8 or C3–C8, an HMBC experiments were used. Indeed, the common correlations of H6 (129.3 ppm, 120.4 ppm) and the only naphthyl singulet (H1 or H8) confirmed its position on C1 (also visible by NOESY). All these data validated the addition on both C8 and C3 positions (See Supplementary data).

This last example led us to question about the amine influence on regioselectivity in basic conditions that has never been previously described. Compound **14** was also engaged in the same reaction conditions and as awaited, no reaction (even at ortho to amino position) was observed (Scheme 2).

Altogether, these results allow concluding about the necessity of a hydroxyl substituent for regioselective substitution in basic conditions and warned us about the possible reactivity of ortho amino position even in basic condition when the naphthyl moiety is both hydroxyl and amino substituted. We proceeded to the synthesis of compounds **4** and **7** by coupling various diazonium **2** to compound **3**. Compounds were purified by classical precipitation at pH 2 in a water/ethanol mixture. Alternative preparative HPLC purification was used when no precipitate had formed or when the purity was not sufficient; this second purification highly impacted global yields as shown in Table 1.

Interestingly, when coupling 4-carboxy or 4-bromo-tetrafluorophenyl diazonium **2** to naphthyl moiety **3**, an aromatic substitution occurred and one ortho-fluorine substituent was replaced by a hydroxyl group in **4f** and **4n**. This substitution has only been observed in few cases mostly without further explanation and only with phase transfer agent in organic solvent. Marriot et al. postulated an assistance of a sodium ion that could help the hydroxyl group attack where Feldman et al. postulated an enhance reactivity of hydroxyl ion in organic solvent [25,26]. It should be also noted that no substitution occurred when stirring tetrafluoro-aniline alone at pH 9 (data not shown). Regarding the regioselectivity, as previously observed for compound **13**, (Scheme 2), some di-substitutions affording compounds **16**, were observed but only when coupling highly reactive diazonium **2** (i.e. ortho substituted by electro-withdrawing group (R_1 = -F, compound **2d**, $R_1 = -CF_3$, compound **2e**) with compound **3**. (Supplementary Scheme 1) As observed with compound **13**, the second addition occurred at ortho position of the aniline substituent and not at C1.

2.2. Pharmacology

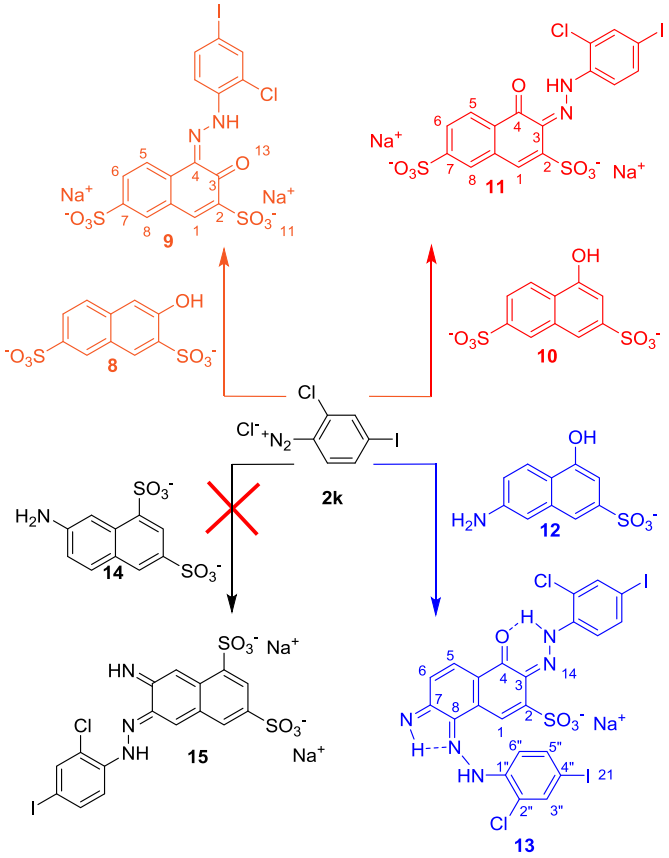
All synthesized compounds **4** and **7** were then evaluated for their ability to inhibit ^{3}*H*-glutamate uptake into isolated vesicles from rat brain. As previously described, inhibition was measured by

Table 1
Yields of compounds 4 and 7 over the two steps.

Entry	*R*1	*R*2	*R*3	*R*4	*R*5	Yields
4a[a]	CH_3	H	COOH	H	H	37%
4b[a]	H	H	COOH	H	H	80%
4c[a]	Cl	H	COOH	H	H	84%
4d[a,b]	F	H	COOH	H	H	12%
4e[a,b]	CF_3	H	COOH	H	H	13%
4f[a]	F	F	COOH	F	OH	52%
4g[a]	OCH_3	H	COOH	H	H	85%
4h[a]	CH_3	H	COOH	H	CH_3	74%
4i[a]	CH_3	H	I	H	H	18%
4j[a]	H	H	Br	H	H	24%
4k[a]	Cl	H	I	H	H	6%
4l[a,b]	F	H	I	H	H	3%
4m[a,b]	CF_3	H	I	H	H	2%
4n[a]	F	F	Br	F	OH	74%
4o[a]	OCH_3	H	Br	H	H	20%
4p[a]	CH_3	H	Br	H	CH_3	24%
7a[a]	CH_3	H	[c]	H	H	2%
7b[a]	H	H	[c]	H	H	25%
7c[a]	Cl	H	[c]	H	H	40%
7d[a,b]	F	H	[c]	H	H	2%
7e[a,b]	CF_3	H	[c]	H	H	42%
7f[a,b]	F	F	[c]	F	F	6%

[a] Purified by precipitation.
[b] Purified by HPLC.
[c] Substituted phenyl as described in Scheme 1.

quantification of tritiated glutamate uptake by purified vesicles [7,27]. Compounds were firstly examined for their capacity to block glutamate uptake at 20 μM level (Tables 2and 3). IC_{50}'s of derivatives **4** and **7** that displayed more than 60% of inhibition at 20 μM, were next determined. CCCP (vATPase inhibitor) and TB (competitive inhibitor (IC_{50} = 0.1 μM)) were used as negative controls (100% of inhibition). No Inhibitor was added for positive control (i.e. tritiated glutamate is typically accumulated in vesicles: 0% of inhibition), allowing the complete inhibitory calibration.



Scheme 2. Regioselective addition of diazonium **2k** to compounds **8**, **10**, **12**, and **14**.[a]Reagents and conditions: NaOH 10%, pH = 9, 0–2 °C.

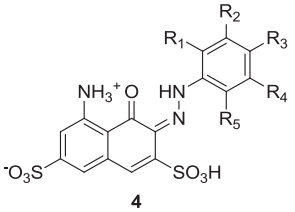
A first series of monoazo-dyes **4**, **9**, **11** and **13** was tested and revealed that most of **4** were able to reduce vesicular glutamate uptake at 20 μM (Table 2) whereas **9**, **11** and **13** were inactive. These results demonstrate that the di-sulfonic naphthalene moiety bearing an amino and a hydroxyl group builds up a core that displays increased potency as compared to the di-carboxylic quinolines DCQs (Fig. 1) [28]. Interestingly when best analogs such as DCQ1 described by Carrigan et al. [12] inhibit VGLUT with an IC_{50} around 40 μM, many of our easily accessible compounds appear promising at 20 μM (**4i**, **4p**, **4m**) (Tables 2and 3).

The first observation allows concluding that *p*-carboxylic acid compounds appear mostly less active than corresponding *p*-halogeno substituted analogues. Only **4e**, the 2′-CF3, 4′-COOH analog is found among the most potent inhibitors, with an IC_{50} of 6.8 μM. The weaker activity of these compounds may be correlated to the negative charge of carboxylate function and not to its electro-withdrawing character. Indeed, compounds **4i** and **4m** bearing respectively *ortho*-methyl, *para*-iodo and *ortho*-trifluoromethyl, *para*-iodo substituent showed similar activity at 20 μM (76 and 83%) despite the opposite character of their ortho substituent. Again, the *ortho*-methoxy *para*-bromo **4o** and ortho -fluoro; *para*-iodo **4l** similarly blocked glutamate uptake (42 and 46% at 20 μM). The same result is observed when comparing activity of **4g** (*ortho*-methoxy; *para*-carboxylic acid) with **4d** (*ortho*-fluoro; *para*-carboxylic acid) both moderately active (respectively 25% and 18% of inhibition at 20 μM). A hydrophobic counterpart in *para* position appears more beneficial as observed for halogenated derivatives. Similarly to EB and CSB, replacement of a methyl group (**4i**, IC_{50} = 2.1 μM) by a methoxy (**4o**, IC_{50} > 20 μM) leads to a marked decrease of activity. The relatively moderate activity of methoxy derivatives appears to be independent of substituent electronic properties. As a matter of fact, electro-withdrawing substituted compounds like mono-fluorinated (R_1 = F) **4l** or chlorinated (R_1 = Cl) **4k** as well as electro-donating substituted derivatives (R_1 = OMe) **4o** inhibit glutamate uptake with similar potency at 20 μM (46, 45% and 42% inhibition respectively). A plausible explanation for the reduced inhibitory activity could be the limited volume available around R1 position to interact with VGLUTs. The bulk (Van der walls) of substituent seems to affect directly inhibitory properties and to be optimal with a methyl group. Compounds **4i** and **4p** are the most active derivatives (IC_{50} = 2.1 μM for both). An increase of the substituent size diminishes the potency of compounds e.g. **4o** compared to **4i** (42% of inhibition at 20 μM compared to 83% for **4i**). On the other hand reducing the volume decreases also inhibitory efficacy of compounds (e.g. 83% and 46% inhibition for **4m** and **4l**). Moreover, compound **4n** appears one of the less active compounds of this series probably because of the presence of a polar substituent (hydroxyl) in the hydrophobic environment.

Additionally the azo-hydrazo tautomer equilibrium may also influence the inhibitory capacity of **4** but according to spectroscopic data all compounds **4** are found only in the hydrazo form ($\delta^{13}C$ (C4) = 180 ppm) [23] and no azo form have been detected in NMR. As described previously for DCQs, a hydrophobic substituent at *para*-phenyl position of compounds is favorable for inhibition; we then decided to increase the size of compounds at this position by adding one functionalized phenyl ring.

The series of compounds **7** (Scheme 1) may be viewed as biphenyl analogs of **4** but also as TB derivatives shortened by one naphthyl. As for previous series, we focused our effort on the biaryl

Table 2
VGLUT inhibitory activity of compounds **4**.[a]



4

Entry	R_1	R_2	R_3	R_4	R_5	% Inhibition (at 20 μM)	IC_{50} (μM)
4a	CH_3	H	COOH	H	H	15	/
4b	H	H	COOH	H	H	15	/
4c	Cl	H	COOH	H	H	55	/
4d	F	H	COOH	H	H	18	/
4e	CF_3	H	COOH	H	H	76	6.8 ± 1.8
4f	F	F	COOH	F	OH	18	/
4g	OCH_3	H	COOH	H	H	25	/
4h	CH_3	H	COOH	H	CH_3	5	/
4i	CH_3	H	I	H	H	83	2.1 ± 1.6
4j	H	H	Br	H	H	33	/
4k	Cl	H	I	H	H	45	/
4l	F	H	I	H	H	46	/
4m	CF_3	H	I	H	H	83	5.8 ± 1.2
4n	F	F	Br	F	OH	39	/
4o	OCH_3	H	Br	H	H	42	/
4p	CH_3	H	Br	H	CH_3	77	2.1 ± 1.3

[a] All experiment have been assayed at least in triplicate.

substituents and kept an azo-link between the bi-aryl and naphthyl moieties (Fig. 1). In contrast to what was observed for smaller structures, it appears that the steric bulk is not the only factor influencing inhibitory properties of compounds (Table 3).

Indeed, direct comparison between uptake activities and van der Walls volumes does not explain the activity variation. Results with the optimal substituent size characterized previously, provide a compound with moderate inhibitory activity (**7a**, R_1 = CH3); and compounds **7c** (R_1 = Cl) or **7d** (R_1 = F), bearing respectively larger and smaller groups, are significantly more potent (IC_{50} = 1.6 and 4.8 μM). In contrast to their analogs **4**, biphenyl substituted naphthyl **7** activities seems to be correlated to the electronic character of biphenyl substituent. Indeed, electro-withdrawing substituted **7c**, **7d**, **7e**, **7f** showed significant inhibition where electro-donating analogs **7a**, **7b** appears quite weaker. These results question about the strict equivalent position of this series compared to the previous and confirm the relative flexibility of the binding site of VGLUT due to its physiological transport function and mechanism. In this group of compounds, a compromise between steric bulk and electronic properties appears crucial for activity. Compared to the previous one, chlorinated analog **7c** is more potent than the methylated (**7a**) or the unsubstituted (**7b**) which are less active than the poly-fluorinated compound **7f**.

In the two series of inhibitors **4** and **7**, CF_3 substituted analogs display particular biological properties and are all found to be potent (Tables 2 and 3). Indeed, among the "half" TB analogs *para*-substituted with a carboxylic acid, only **4e** shows significant inhibitory activity all other analogs being much less active. As for other *para*-halogenated compounds **4m** is most potent with an IC_{50} value of 5.8 μM. The best analog remains the bi-aryl substituted compound **7e**. Another explanation could be the conformation adopted by compounds particularly between azo and phenyl/biphenyl parts but preliminary studies didn't allow to conclude (Supplementary Table 2).

Best compounds have also been evaluated for their ability to interfere with Vesicular Monoamine Transporter uptake. Indeed, even if the parent drug TB has been characterized as a competitive inhibitor [3] other dyes such as Rose Bengal can modulate the transport of monoamine, probably by indirect interaction (potential or proton gradients are crucial and could be targeted by non-competitive drugs) [10]. Moreover, purified vesicles used for uptake experiments are extracted from a brain region where both VMAT2 and VGLUTs highly co-localize. [29] Compounds **4** and **7** show a good selectivity because no effect on dopamine uptake has been detected at 200 μM (data not shown). These results confirm that these analogs did not interact with ATPase or with other proteins implicated in proton uptake. On the other hand, we also verified that glutamate competitive transporter inhibitors did not interact with glutamate receptors such as mGlu1-8 receptors. We evaluated the activity of the best analogs **4i** and **7c** on all receptor subtypes in order to cover the wide range of glutamate affinity for mGluRs (1 mM to 1 μM). As expected, no significant agonist or antagonist effect has been characterized at 200 μM in cell based assays showing a particular non cytotoxicity of these azo dyes as previously described [30,31].

3. Conclusion

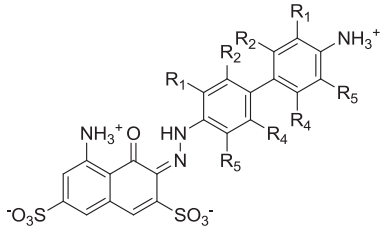
In conclusion an SAR study in a TB derived series allows the characterization of key features required for potent VGLUT uptake inhibition. A comparison between DCQ and naphthyl analogs confirms the importance of an amino group in this scaffold, whereas the positions of negative charges are not crucial as underlined by similar activity of TB and EB. More precisely, this study has disclosed strategic features for VGLUT inhibitor design such as volume limitation for biaryl functionalization or the detrimental effect of a negative charge. In the present study, we have synthesized a mini chemical library of small VGLUTs ligands with improved synthetic methodology, yields and biological potencies. Once the synthetic experimental conditions were optimized, access to various structures was straightforward and allowed a rapid characterization of key features for VGLUT uptake inhibition. The preliminary observations made by Carrigan et al. [12] have been confirmed and completed. Among these characteristics, bi-aryl derivatives and 8-hydrolylated substituent are needed for improved potencies. Our results should be considered in the context of the very limited pharmacology of VGLUTs [3,32]. Moreover, the affinity of glutamate for VGLUTs is very low (Km = 1–3 mM) whereas the best structural analog interact with VGLUT with an IC_{50} of 0.2 mM [3]. DCQs, which have been designed for the same purpose as **4** and **7**, displayed IC_{50}'s of 40 and 95 μM [12]. Our compounds, in particular **7c**, **7e**, **4i** and **4p**, are active in the micromolar range, and are thus one of the best compromise between activity and selectivity for pharmacological VGLUT inhibition. They pave the way as pharmacological tools to the regulation of glutamate neurotransmission, a crucial player in a large number of CNS pathologies.

4. Experimental section

4.1. General

All chemicals and solvents were purchased from commercial suppliers (Acros, Aldrich, Alpha Aesar, Apollo Scientific) and used as received. Most solvents were purchased from Carlo Erba-SDS. Solvents for reactions were dried on 4 Å molecular sieves (Carlo Erba – SDS) or were distillated. All reactions were monitored by thin layer chromatography (TLC) on precoated silica on aluminum (Merck 60 F_{254}, aluminum sheets) and revealed by UV. Silica gel chromatography was performed with Silica gel 60 Merck®, 40–63 μm.

Table 3
VGLUT inhibitory activity of compounds **7**.[a]



7

Entry	*R*1	*R*2	*R*4	*R*5	% Inhibition (at 20 μM)	IC_{50} (μM)
7a	CH3	H	H	H	52	/
7b	H	H	H	H	25	/
7c	Cl	H	H	H	88	1.6 ± 1.1
7d	F	H	H	H	74	4.8 ± 1.6
7e	CF3	H	H	H	98	1.8 ± 1.5
7f	F	F	F	F	96	3.3 ± 1.4

[a] All experiment have been assayed at least in triplicate.

Reversed phase TLC were performed using RP-18 F_{254} plates (Merck®), and reversed-phase chromatography with silica gel 100C_{18}-reversed phase (Fluka®). Compounds were purified on a Gilson analytical instrument with a 321 pump, eluted peaks were detected by a UV–vis 156 detector and retention times are reported in minutes. System was equipped with a Macherey–Nagel Nucleodur® 100-10C18 (250 mm × 32 mm, 10 μm). ^1H (250.13 or 400.13 or 500.16 MHz), ^{13}C (62.9 or 100.62 or 125.78 MHz) and ^{19}F (376.49 MHz) NMR spectra were respectively recorded on an ARX 250 or an Avance II 400 Bruker or an Avance II 500 Bruker spectrometer.

Chemical shifts (δ) are given in δ (ppm) with reference to residual ^1H or ^{13}C of deuterated solvents, respectively CD_3OD 3.31, 49.0; D_2O 4.80; $CDCl_3$ 7.24, 77.00; $(CD_3)_2CO$ 2.05, 29.84 and 206.26 or $(CD_3)_2SO$ 2.50, 39.52). Mass spectra (MS) were recorded with an LCQ-advantage (ThermoFinnigan) mass spectrometer with positive (ESI^+) or negative (ESI^-) electrospray ionization (ionization tension 4.5 kV, injection temperature 240 °C). HPLC-MS analyses were performed on a Thermo Finnigan LCQ Advantage Instrument as described above, equipped for HPLC with a Macherey–Nagel Nucleosil® 100-5 C18 (125 mm × 4 mm, 4 μm). Products were eluted as the same manner as described above.

HPLC analyses were carried out on two devices: a Gilson analytical instrument with a 321 pump, column temperature of Nucleosil® columns was controlled with an Igloo-CIL Peltier effect thermostat or column oven Shimadzu CTO-10A, eluted peaks were detected by a UV–vis 156 detector and retention times are reported in minutes. A Gilson analytical instrument with a 306/307 pump, column temperature of Nucleosil® columns was controlled with an Igloo-CIL Peltier effect thermostat or column oven Shimadzu CTO-10A, eluted peaks were detected by a UV–vis 155 detector and retention times are reported in minutes. Purity of the tested compounds was established by analytical HPLC-MS, by HPLC or by elementary analysis and was at least 95%. UV spectra were performed Saphire spectrofluorimeter in pure water. Modeling figure has been made using discovery Studio 2.5 software.

4.2. General procedure

The following gradients were used preparative (Gradient P1–P2) and analytical (Gradient A) HPLC: Gradient P1 is using solvent A: ammonium acetate buffer 0.05 M, solvent B: acetonitrile, 80% A for 4 min, linear increase from 20 to 50% B between 4 and 40 min, 50% A – 50% B from 40 min to 44 min, linear decrease from 50 to 20% B between 44 and 46 min, 80% A between 46 and 56 min. Flow: 8 mL min^{-1}. $\lambda_1 = 254$ nm, $\lambda_2 = 550$ nm. Gradient P2 is using solvent A: ammonium acetate buffer 0.05 M, solvent B: acetonitrile, 80% A for 4 min, linear increase from 20 to 50% B between 4 and 32 min, 50% A – 50% B from 32 min to 47 min, linear decrease from 50 to 20% B between 47 and 50 min, 80% A between 50 and 55 min. Flow: 8 mL min^{-1}. $\lambda_1 = 254$ nm, $\lambda_2 = 550$ nm. Gradient A is using solvent A: water-formic acid 1‰, solvent B: acetonitrile 90% A for 5 min, linear increase from 10 to 60% B between 5 and 22 min, 40% A – 60% B from 22 min to 30 min, linear decrease from 60 to 10% B between 30 and 32 min, 90% A between 32 and 36 min. Flow: 0.75 mL min^{-1}. λ1 = 254 nm, λ2 = 550 nm. Prior to use: 5-Amino-4-hydroxynaphthalene-2,7-disulfonic acid monosodium salt was recrystallized three times at pH = 3 in a mixture of EtOH/H_2O –1/1.

4.2.1. General procedure A

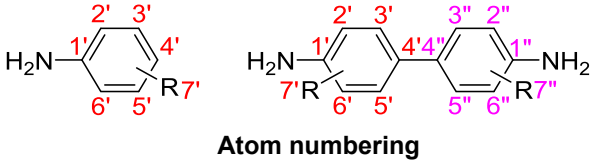
Aniline derivative (1 eq) was mixed with iced water and/or absolute EtOH (2 mL by mmol of reagent) and stirred at 0 °C then hydrochloric acid 37% (4 eq) was added dropwise rapidly. Sodium nitrite (1.1 eq or 1.3 eq aniline substituent depending) was added solid or in solution (H_2O/EtOH 1 M) and the reaction mixture was stirred at 0–2 °C for 30 min. The solvent was removed carefully under reduced pressure without heating and can be used without further purification.

4.2.2. General procedure B

5-Amino-4-hydroxynaphtalene-2,7-disulfonic acid sodium salt (0.9 eq) was dissolved in iced water (5 mL by mmol of reagent) then pH was adjusted to 9 by adding 30% NaOH solution. The diazonium salt was added to the naphthalene moiety and pH maintained at 9 by addition of 10% NaOH solution. After complete addition of the diazonium salt to the 5-amino-4-hydroxynaphthalene-2,7-disulfonic acid monosodium salt, stirring was maintained at 0 °C for 2 h. Degraded diazonium was removed after extraction with EtOAc and water soluble diazo compounds were recrystallized three times from EtOH/(H_2O; pH = 2) (1/1). All organic impurities were then extracted by washing with small portions of Et_2O. The precipitated compounds were dried under vacuum.

4.2.3. General procedure C

Benzidine derivative (1 eq) was mixed with iced water and/or absolute EtOH (2 mL by mmol of reagent) and stirred at 0 °C then hydrochloric acid 37% (8 eq) was added dropwise rapidly. Sodium nitrite (2.2 eq or 2.6 eq aniline substituent depending) was added in solid or in solution (H_2O/EtOH 1 M) below 2 °C and reaction mixture was stirred at 0–2 °C for 30 min. Naphtalenesulfonique compound (1.8 eq) was dissolved in iced water (10 mL by mmol of reagent) then pH was adjusted to 9 by adding NaOH 30% solution. The diazonium salt was coupled in alkaline media (pH 9) below 2 °C by adding NaOH 10% solution. After complete addition of the diazonium salt to the 5-amino-4-hydroxynaphthalene-2,7-disulfonic acid monosodium salt, stirring was maintained at 0 °C for 2 h. Degraded diazonium was removed after extraction with EtOAc and water soluble diazo compounds were recrystallized three times from EtOH/(H_2O; pH = 2) (1/1). All organic impurities were then extracted by washing with small portion of Et_2O. The precipitated compounds were dried under vacuum.



4.2.3.1. 4-Amino-3,5-dimethylbenzoic acid, ***1h***. 3,5-dimethyl-4-nitrobenzoic acid (976 mg, 5 mmol) was suspended in water (20 mL) under vigorously stirring for 30 min, until mixture was homogenized); Na_2CO_3 (300 mg, 2.8 mmol) and zinc dust (3 g, 30 mmol) were added to the solution then the mixture was heated fat 30 °C and HCl conc. (10 mL) was added dropwise over 20 min. Thereafter, the solution was filtered, and the filter cake was washed twice with water (2 × 7 mL). To remove unreacted 3,5-dimethyl-4-nitrobenzoic acid, the filtrate was washed twice with ethyl acetate (2 × 40 mL). The aqueous layer was adjusted to pH 9.35 by addition of saturated Na_2CO_3 solution (35 mL), and the precipitated ZnOH was filtered off. Once more, the filter cake was washed twice with 5% $NaHCO_3$ (2 × 10 mL). To remove by products, the filtrate was washed twice with EtOAc (2 × 80 mL). Subsequently, the aqueous layer was adjusted to pH 3.5 by addition of 1 N HCl (~120 mL). Finally, the solution was extracted five times with ethyl acetate (5 × 80 mL). The combined organic layers were dried with $MgSO_4$ and evaporated dryness. Compound was obtained as a clear brown solid in 76% yield (629 mg). 1H NMR (500 MHz, CD_3CN): δ 8.94 (brs, 1H, OH); 7.55 (s, 2H, H3′, H5′); 4.52 (brs, 2H, NH_2); 2.15 (s, 6H, H7′) ^{13}C NMR (126 MHz, CD_3CN): δ 168.5 (C8′); 149.9 (C1′); 131.0 (C3′, C5′); 122.4(C4′); 121.4 (C2′,C6′); 17.9 (C7′). MS (ESI)+: *m*/*z* 166 [M + H]+.

4.2.3.2. 4-carboxy-2-Methylbenzenediazonium chloride, ***2a***. Following the general procedure A, 4-Amino-3-methylbenzoic acid (453 mg, 3 mmol), HCl 37% (1 mL, 12 mmol), NaNO2 in solution (227 mg, 2.3 mL), solvent H_2O/EtOH. Compound was obtained as a white powder in 100% yield (595 mg). 1H NMR (500 MHz, MeOD): δ 8.69 (d, *J* = 8.5 Hz, 1H, H6′); 8.39 (d, *J* = 1.2 Hz, 1H, H3′); 8.31 (dd, *J* = 8.5 Hz, *J* = 1.2 Hz, 1H, H5′); 2.87 (s, 3H, H7′).

4.2.3.3. 4-carboxybenzenediazonium chloride ***2b***. Following the general procedure A, 4-Aminobenzoic acid (685 mg, 5 mmol), HCl 37% (1.67 mL, 20 mmol), $NaNO_2$ in solid (380 mg, 5.5 mmol), solvent H_2O. Compound was obtained as a white powder in 100% yield (925 mg). ^1H NMR (500 MHz, MeOD): δ 8.87 (d, *J* = 8.8 Hz, 2H, $H_{2'}$—$H_{6'}$); 8.39 (d, *J* = 8.8 Hz, 2H, $H_{3'}$—$H_{5'}$)

4.2.3.4. 4-carboxy-2-fluorobenzenediazonium chloride, ***2d***. Following the general procedure A, 4-Amino-3-fluorobenzoic acid (620 mg, 4 mmol), HCl 37% (1.33 mL, 16 mmol), $NaNO_2$ in solid (303 mg, 4.4 mmol), solvent H_2O. Compound was obtained as a white powder in 95% yield (770 mg). ^1H NMR (500 MHz, MeOD): δ 8.74 (dd, *J* = 8.5 Hz, *J* = 5.6 Hz, 1H, $H_{6'}$); 8.32 (d, *J* = 9.8 Hz, 1H, $H_{3'}$); 8.25 (d, *J* = 8.5 Hz, 1H, $H_{5'}$).

4.2.3.5. Iodobenzenediazonium chloride, ***2j***. Following the general procedure A, 4-iodoaniline (1095 mg, 5 mmol), HCl 37% (1.67 mL, 20 mmol), $NaNO_2$ in solide (380 mg, 5.5 mmol), solvent H_2O. Compound was obtained as a white powder in 100% yield (1330 mg). ^1H NMR (500 MHz, MeOD): δ 8.81 (d, *J* = 8.8 Hz, 2H, $H_{2'}$, $H_{6'}$); 8.34 (d, *J* = 8.8 Hz, 2H, $H_{3'}$, $H_{5'}$)

4.2.3.6. 4-bromo-2-fluorobenzenediazonium chloride, ***2l***. Following the general procedure A, 4-Bromo-2-fluoroaniline (760 mg, 4 mmol), HCl 37% (1.33 mL, 16 mmol), $NaNO_2$ in solution (3 mL, 3 mmol), solvent H_20—EtOH. Compound was obtained as a white powder in 100% yield (810 mg). ^1H NMR (500 MHz, MeOD): δ 8.62 (dd, *J* = 9 Hz, *J* = 6.3 Hz, 1H, $H_{6'}$); 8.35 (dd, *J* = 9 Hz, *J* = 1.8 Hz, 1H, $H_{5'}$); 8.08 (d, *J* = 9 Hz, 1H, $H_{3'}$).

4.2.3.7. 2,4-Dinitrobenzenediazonium chloride, ***2q***. Following the general procedure A, 2,4-Dinitroaniline (366 mg, 2 mmol), HCl 37% (0.7 mL, 8 mmol), $NaNO_2$ in solution (2.6 mL, 2.6 mmol), Solvent EtOH. Compound was obtained as a white powder in 100% yield (460 mg). ^1H NMR (500 MHz, MeOD): δ 9.61 (d, *J* = 2.9 Hz, 1H, $H_{3'}$); 8.96 (dd, *J* = 9.4 Hz, *J* = 2.9 Hz, 1H, $H_{5'}$); 7.97 (d, *J* = 9.4 Hz, 1H, $H_{6'}$).

4.2.3.8. (E)-5-ammonio-3-(2-(4-carboxy-2-methylphenyl)hydrazono)-4-oxo-3,4-dihydronaphthalene-2,7-disulfonate, ***4a*** *(LSP9-2129).* Following the general procedure B, corresponding diazonium salt (1.4 mmol) was added to 5-Amino-4-hydroxynaphthalene-2,7-disulfonic acid (445 mg, 1.3 mmol). Compound was obtained as a dark blue powder in 37% yield (300 mg). ^1H NMR (500 MHz, DMSO): δ 15.57 (s, 1H, NH); 8.04 (d, *J* = 8.4 Hz, 1H, $H_{6'}$); 7.85 (s, 1H, $H_{3'}$); 7.83 (d, *J* = 8.4 Hz, 1H, $H_{5'}$); 7.33 (s, 1H, H_1); 7.09 (s, 1H, H_6); 6.87 (s, 1H, H_8); 2.42 (s, 3H, $H_{7'}$). ^{13}C NMR (126 MHz, DMSO): δ 181.9 (C_4); 166.3 ($C_{7'}$); 153.7 (C_7); 153.5 (C_2); 144.8 ($C_{4'}$); 142.9 (C_5); 135.9 (C_9); 130.8 ($C_{3'}$); 130. 3 (C_3); 129.1 ($C_{2'}$); 128.3 ($C_{5'}$); 124.4 (C_1); 123.6 ($C_{1'}$); 113.2 ($C_{6'}$); 114.3 (C_8); 113.1 (C_6); 111.5 (C_{10}); 17.05 ($C_{8'}$). Elemental analysis found (calcd.) (+NaCl; +Na+; $+3H_3O^+$): C 33.99 (34.05); H 3.80 (3.65); N 6.79 (6.62); S 10.15 (10.10). HPLC (Gradient A) t_R = 8.20 min. MS (ESI)$^-$: *m*/*z* 480.1 $[M - H]^-$. UV vis λ_{max} = 543 nm.

4.2.3.9. (E)-5-Ammonio-3-(2-(4-carboxyphenyl)hydrazono)-4-oxo-3,4-dihydronaphthalene-2,7-disulfonate, ***4b*** *(LSP9-2177).* Following the general procedure B, corresponding diazonium salt (1.9 mmol) was added to 5-Amino-4-hydroxynaphthalene-2,7-disulfonic acid (581 mg, 1.7 mmol). Compound was obtained as a dark blue powder in 10% yield (92 mg). ^1H NMR (500 MHz, DMSO): δ 15.24 (s, 1H, NH); 7.94 (d, *J* = 8.8 Hz, 2H, $H_{3'}$—$H_{5'}$); 7.72 (d, *J* = 8.8 Hz, 2H, $H_{2'}$—$H_{6'}$); 7.30 (s, 1H, H_1); 7.08 (s, 1H, H_6); 6.86 (s, 1H, H_8). ^{13}C NMR (126 MHz, DMSO): δ 181.9 (C_4); 166.8 ($C_{7'}$); 153.9 (C_7); 153.4 (C_2); 146.4 ($C_{4'}$); 142.8 (C_5); 136.2 (C_9); 130.7 ($C_{3'}$—$C_{5'}$); 130. 3 (C_3); 125.6 ($C_{1'}$); 123.7 (C_1); 115.7 ($C_{2'}$—$C_{6'}$); 113.7 (C_8); 112.5 (C_6); 111.5 (C_{10}). Elemental analysis found (calcd.) ($+Na^+$; $+3H_3O^+$): C 37.81 (37.36); H 3.41 (3.87); N 7.46 (7.86); S 11.02 (11.74). HPLC (Gradient A) t_R = 5.65 min. MS (ESI)$^-$: *m*/*z* 465.9 $[M - H]^-$. UV vis λ_{max} = 534 nm.

4.2.3.10. (E)-5-Ammonio-3-(2-(4-carboxy-2-chlorophenyl)hydrazono)-4-oxo-3,4-dihydronaphthalene-2,7-disulfonate, ***4c*** *(LSP9-2192).* Following the general procedure B, corresponding diazonium salt (1.6 mmol) was added to 5-Amino-4-hydroxynaphthalene-2,7-disulfonic acid (479 mg, 1.4 mmol). Compound was obtained as a dark blue powder in 84% yield (630 mg). ^1H NMR (500 MHz, DMSO): δ 15.24 (s, 1H, NH); 8.11 (d, *J* = 8.6 Hz, 1H, $H_{6'}$); 8.0 (d, *J* = 1.6 Hz, 1H, $H_{3'}$); 7.93 (dd, *J* = 8.6 Hz, *J* = 1.6 Hz, 1H, $H_{5'}$); 7.36 (s, 1H, H_1); 7.12 (s, 1H, H_6); 6.88 (s, 1H, H_8). ^{13}C NMR (126 MHz, DMSO): δ 182.2 (C_4); 165.9 ($C_{7'}$); 154.2 (C_7); 153.8 (C_2); 142.9 (C_5); 142.8 ($C_{4'}$); 136.0 (C_9); 131.7 (C_3); 130.4 ($C_{3'}$); 129.4 ($C_{5'}$); 126.2 ($C_{1'}$); 125.2 (C_1); 119.1 ($C_{2'}$); 116.1 ($C_{6'}$); 114.2 (C_8); 113.2 (C_6); 111.0 (C_{10}). Elemental analysis found (calcd.) ($+Na^+$; $+3H_3O^+$): C 37.81 (37.36); H 3.41 (3.87); N 7.46 (7.86); S 11.02 (11.74). HPLC (Gradient A) t_R = 8.36 min. MS (ESI)$^-$: *m*/*z* 500.0 $[M - H]^-$. UV vis λ_{max} = 536 nm.

4.2.3.11. (E)-5-Ammonio-3-(2-(4-carboxy-2-fluorophenyl)hydrazono)-4-oxo-3,4-dihydronaphthalene-2,7-disulfonate, ***4d*** *(LSP9-2135).* Following the general procedure B, corresponding diazonium salt (1.4 mmol) was added to 5-Amino-4-hydroxynaphthalene-2,7-disulfonic acid (445 mg, 1.3 mmol). The crude product was purified by preparative HPLC gradient P2. Compound *LSP9-2135* was the first eluted and obtained as a violet powder in 12% yield (80 mg). ^1H NMR (500 MHz, DMSO): δ 15.38 (s, 1H, NH); 8.02 (t, *J* = 8.3 Hz, 1H, $H_{6'}$); 7.80 (dd, J_1 = 8.3 Hz, J_2 = 1.5 Hz, 1H, $H_{5'}$); 7.75 (dd, J_1 = 11.8 Hz, J_2 = 1.5 Hz, 1H, $H_{3'}$); 7.33 (s, 1H, H_1); 7.10 (d, *J* = 1.5 Hz, 1H, H_6); 6.87 (d, *J* = 1.5 Hz, 1H, H_8). ^{13}C NMR

(126 MHz, DMSO): δ 182.3 (C_4); 166.7 ($C_{7'}$); 153.9 (C_7); 153.8 (C_2); 149.2 ($C_{2'}$); 148.1 ($C_{4'}$); 142.5 (C_5); 136.2 (C_9); 131.4 (C_3); 126.7 ($C_{5'}$); 124.8 (C_1); 116.4 ($C_{6'}$); 116.2 ($C_{3'}$); 114.2 (C_8); 113.1(C_6); 111.4 (C_{10}). Elemental analysis found (calcd.) ($+2Na^+$; $+1Cl^-$; $3H_3O^+$): C 33.12 (32.78); H 3.17 (3.24); N 6.26 (6.75). HPLC (Gradient A) t_R = 8.77 min. MS (ESI)$^-$: *m/z* 484.1 [M − H]$^-$. UV vis λ_{max} = 532 nm.

4.2.3.12. (E)-5-Ammonio-3-(2-(4-carboxy-2-(trifluoromethyl) phenyl)hydrazono)-4-oxo-3,4-dihydronaphthalene-2,7-disulfonate, ***4e*** *(LSP9-3041).* Following the general procedure B, corresponding diazonium salt (1.4 mmol) was added to 5-Amino-4-hydroxynaphthalene-2,7-disulfonic acid (445 mg, 1.3 mmol). The crude product was purified by preparative HPLC gradient P2. Compound was the first eluted and obtained as a violet powder in 13% yield (98 mg). ^{1}H NMR (500 MHz, DMSO): δ 15.82 (s, 1H, NH); 8.16 (m, 3H, $H_{3'}$, $H_{5'}$, $H_{6'}$); 7.32 (s, 1H, H_1); 7.10 (s, 1H, H_6); 6.86 (s, 1H, H_8). ^{13}C NMR (126 MHz, DMSO): 182.0 (C_4); 172.3 ($C_{7'}$); 154.2 (C_7); 153.7 (C_2); 142.6 ($C_{4'}$); 141.1 ($C_{5'}$); 136.1 ($C_{1'}$); 134.1 (C_5); 131.1 (C_9); 126.7 ($C_{3'}$); 125.4 (C_3); 124.5 ($C_{6'}$); 117.0 ($C_{7'}$); 116.5 (C_1); 114.1 (C_8); 113.5 ($C_{2'}$); 112.9 (C_6); 111.0 (C_{10}); Elemental analysis found (calcd.) ($+2NaCl^+$; $+H_2O$): C 32.35 (32.30); H 2.00 (1.96); N 6.27 (6.28); S 9.60 (9.58). HPLC (Gradient A) t_R = 11.80 min. MS (ESI)$^-$: *m/z* 534.1 [M − H]$^-$. UV vis λ_{max} = 529 nm.

4.2.3.13. (E)-5-Ammonio-3-(2-(4-carboxy-2,3,5-trifluoro-6-hydroxyphenyl)hydrazono)-4-oxo-3,4-dihydronaphthalene-2,7-disulfonate, ***4f*** *(LSP9-3031).* Following the general procedure B, corresponding diazonium salt (1.4 mmol) was added to 5-Amino-4-hydroxynaphthalene-2,7-disulfonic acid (410 mg, 1.2 mmol). Compound was obtained as a violet powder in 52% yield (335 mg). ^{1}H NMR (500 MHz, DMSO): δ 15.48 (s, 1H, NH); 10.60 (s, 1H, OH); 7.3 (s, 1H, H_1); 7.16 (d, *J* = 1.2 Hz, 1H, H_6); 6.9 (d, *J* = 1.2 Hz, 1H, H_8). ^{13}C NMR (126 MHz, DMSO): δ 182.6 (C_4); 161. 6 ($C_{7'}$); 154.6 (C_7); 154.2 (C_2); 145.4 ($C_{5'}$); 140.8 (C_5); 136.4 ($C_{2'}$); 135.8 (C_9); 133.8 ($C_{6'}$); 130.2 (C_3); 125.1 (C_1); 121.4 ($C_{1'}$); 114.9 (C_8); 113.9 (C_6); 110.9 (C_{10}); 106.6 ($C_{4'}$). ^{19}F NMR (376 MHz, DMSO): δ −139.22 (dd, *J* = 8.7 Hz, *J* = 6.4 Hz, 1F, $F_{5'}$); −151.52 (dd, *J* = 23.6 Hz, *J* = 6.4 Hz, 1F, $F_{2'}$); −159.85 (dd, *J* = 23.6 Hz, *J* = 8.7 Hz, 1F, $F_{3'}$). Elemental analysis found (calcd.) ($+Na^+$; $+2\ Cl^-$; $+2H_3O^+$; $+H_2O$): C 29.69 (29.66); H 2.36 (2.34); N 5.97 (6.10). HPLC (Gradient A) t_R = 8.39 min. MS (ESI)$^-$: *m/z* 535.9 [M − H]$^-$. UV vis λ_{max} = 539 nm.

4.2.3.14. (E)-5-Ammonio-3-(2-(4-carboxy-2-methoxyphenyl)hydrazono)-4-oxo-3,4-dihydronaphthalene-2,7-disulfonate, ***4g*** *(LSP9-2137).* Following the general procedure B, corresponding diazonium salt (1.4 mmol) was added to 5-Amino-4-hydroxynaphthalene-2,7-disulfonic acid (445 mg, 1.3 mmol). Compound was obtained as a dark blue powder in 69% yield (480 mg). ^{1}H NMR (500 MHz, DMSO): δ 15.30 (s, 1H, NH); 7.98 (d, *J* = 8.2 Hz, 1H, $H_{6'}$); 7.63 (dd, *J* = 8.2 Hz, *J* = 1.8 Hz, 1H, $H_{5'}$); 7.59 (d, *J* = 1.8 Hz, 1H, $H_{3'}$); 7.29 (s, 1H, H_1); 7.07 (s, 1H, H_6); 6.85 (s, 1H, H_8); 4.04 (s, 3H, $H_{8'}$). ^{13}C NMR (126 MHz, DMSO): δ 181.8 (C_4); 169.9 ($C_{7'}$); 153.8 (C_7); 153.4 (C_2); 146.80 ($C_{2'}$); 142.8 (C_5); 136.2 (C_9); 135.5 ($C_{4'}$); 130.7 (C_3); 126.0 ($C_{1'}$); 123.8 (C_1); 123.1 ($C_{5'}$); 114.8 ($C_{6'}$); 113.7 (C_8); 112.5 (C_6); 111.8 (C_{10}); 111.5 ($C_{3'}$); 56.2 ($C_{8'}$). Elemental analysis found (calcd.) ($+NaCl$; $+Na^+$; $+3H_3O^+$): C 33.99 (34.05); H 3.80 (3.65); N 6.79 (6.62); S 10.15 (10.10). HPLC (Gradient A) t_R = 8.80 min. MS (ESI)$^-$: *m/z* 495.9 [M − H]$^-$. UV vis λ_{max} = 544 nm.

4.2.3.15. (E)-5-Ammonio-3-(2-(4-carboxy-2,6-dimethylphenyl) hydrazono)-4-oxo-3,4-dihydronaphthalene-2,7-disulfonate, ***4h*** *(LSP9-2139).* Following the general procedure B, corresponding diazonium salt (1.4 mmol) was added to 5-Amino-4-hydroxynaphthalene-2,7-disulfonic acid (445 mg, 1.3 mmol). Compound was obtained as a dark blue powder in 74% yield (300 mg). ^{1}H NMR (500 MHz, DMSO): δ 7.68 (s, 2H, $H_{3'}$, $H_{5'}$); 7.34 (s, 1H, H_1); 7.07 (s, 1H, H_6); 6.87 (s, 1H, H_8); 2.62 (s, 6H, $-CH_3$). ^{13}C NMR (126 MHz, DMSO): δ 181.2 (C_4); 167.0 ($C_{7'}$); 153.4 (C_7); 153.2 (C_2); 142.5 ($C_{4'}$); 142.0 (C_5); 136.2 (C_9); 130.5 ($C_{3'}$—$C_{5'}$); 130.4 (C_3); 128.4 ($C_{2'}$—$C_{6'}$); 125.9 (C_1); 123.1 ($C_{1'}$); 113.4 (C_8); 112.1 (C_6); 112.0 (C_{10}); 20.2 ($C_{8'}$—$C_{9'}$) Elemental analysis found (calcd.) ($+NaCl$; $+Na+$; $+4H_3O^+$): C 35.19 (35.00); H 4.30 (4.33); N 6.39 (6.44); S 9.71 (9.84). HPLC (Gradient A) t_R = 8.34 min. MS (ESI)$^-$: *m/z* 494.1 [M − H]$^-$. UV vis λ_{max} = 530 nm.

4.2.3.16. (E)-5-Ammonio-3-(2-(4-iodo-2-methylphenyl)hydrazono)-4-oxo-3,4-dihydronaphthalene-2,7-disulfonate, ***4i*** *(LSP9-2114).* Following the general procedure B, corresponding diazonium salt (5 mmol) was added to 5-Amino-4-hydroxynaphthalene-2,7-disulfonic acid monosodium salt (1591 mg, 4.7 mmol). Compound was obtained as a violet powder in 18% yield (506 mg). ^{1}H NMR (500 MHz, DMSO): δ 15.62 (s, 1H, NH); 7.77 (d, *J* = 8.9 Hz, 1H, $H_{5'}$); 7.65 (s, 1H, $H_{3'}$); 7.62 (d, *J* = 8.9 Hz, 1H, $H_{6'}$); 7.30 (s, 1H, H_1); 7.06 (s, 1H, H_6); 6.87 (s, 1H, H_8); 2.38 (s, 3H, $H_{7'}$). ^{13}C NMR (126 MHz, DMSO): δ 181.4 (C_4); 153.5 (C_7); 153.2 (C_2); 142.7 (C_5); 140.7 ($C_{2'}$); 138.5 ($C_{6'}$); 136.9 (C_9); 135.6 ($C_{3'}$); 130.0 (C_3); 126.9 ($C_{1'}$); 123.0 (C_1); 117.4 ($C_{5'}$); 113.4 (C_8); 112.1 (C_6); 111.7 (C_{10}); 88.0 ($C_{4'}$); 16.0 ($C_{8'}$). Elemental analysis found (calcd.) ($+2Na^+$; $+H_3O^+$): C 32.55 (32.73); H 2.57 (2.61); N 6.70 (6.56); S 10.22 (9.99). HPLC (Gradient A) t_R = 11.78 min. MS (ESI)$^-$: *m/z* 562.0 [M − H]$^-$. UV vis λ_{max} = 543 nm.

4.2.3.17. (E)-5-Ammonio-3-(2-(4-bromophenyl)hydrazono)-4-oxo-3,4-dihydronaphthalene-2,7-disulfonate, ***4j*** *(LSP9-2120).* Following the general procedure B, corresponding diazonium salt (1.4 mmol) was added to 5-Amino-4-hydroxynaphthalene-2,7-disulfonic acid monosodium salt (445 mg, 1.3 mmol). Compound was obtained as a violet powder in 24% yield (545 mg). ^{1}H NMR (500 MHz, DMSO): δ 15.34 (s, 1H, NH); 7.64 (d, *J* = 8.8 Hz, 2H, $H_{2'}$, $H_{6'}$); 7.56 (d, *J* = 8.8 Hz, 2H, $H_{3'}$, $H_{5'}$); 7.26 (s, 1H, H_1); 7.06 (d, *J* = 1.5 Hz, 1H, H_8); 6.84 (d, *J* = 1.5 Hz, 1H, H_6). ^{13}C NMR (126 MHz, DMSO): δ 181.7 (C_4); 153.8 (C_7); 153.6 (C_2); 142.6 (C_5); 118.7 ($C_{2'}$—$C_{6'}$); 137.1 (C_9); 132.6 ($C_{3'}$—$C_{5'}$); 130.9 (C_3); 124.7 ($C_{1'}$); 123.3 (C_1); 113.9 (C_8); 112.6 (C_6); 111.9 (C_{10}); 88.9 ($C_{4'}$). Elemental analysis found (calcd.) ($+2\ Na^+$): C 34.42 (33.93); H 2.96 (2.49); N 7.55 (7.42); S 10.79 (11.32). HPLC (Gradient A) t_R = 10.08 min. MS (ESI)$^-$: *m/z* 499.9 [M−H]$^-$. UV vis λ_{max} = 536 nm.

4.2.3.18. (E)-5-Ammonio-3-(2-(2-chloro-4-iodophenyl)hydrazono)-4-oxo-3,4-dihydronaphthalene-2,7-disulfonate, ***4k*** *(LSP9-2179).* Following the general procedure B, corresponding diazonium salt (1.4 mmol) was added to 5-Amino-4-hydroxynaphthalene-2,7-disulfonic acid (445 mg, 1.3 mmol). Compound was obtained as a dark blue powder in 6% yield (37 mg). ^{1}H NMR (500 MHz, DMSO): δ 15.47 (s, 1H, NH); 7.90 (d, *J* = 1.7 Hz, 1H, $H_{3'}$); 7.83 (d, *J* = 8.7 Hz, 1H, $H_{6'}$); 7.76 (dd, *J* = 8.7 Hz, *J* = 1.7 Hz, 1H, $H_{5'}$); 7.33 (s, 1H, H_1); 7.10 (d, *J* = 1.2 Hz, 1H, H_6); 6.88 (d, *J* = 1.2 Hz, 1H, H_8). ^{13}C NMR (126 MHz, DMSO): δ 181.9 (C_4); 153.9 (C_7); 153.5 (C_2); 142.5 (C_5); 139.3 ($C_{1'}$); 137.1 ($C_{3'}$); 136.8 (C_9); 136.1 ($C_{5'}$); 130.8 (C_3); 124.3 (C_1); 120.4 ($C_{2'}$); 118.6 ($C_{6'}$); 113.9 (C_8); 112.8 (C_6); 111.2 (C_{10}); 87.0 ($C_{4'}$). Elemental analysis found (calc) ($+Na^+$; $+4H_2O$): C 28.20 (28.35); H 2.64 (2.68); N 5.08 (6.20); S 9.96 (9.46). HPLC (Gradient A) t_R = 12.12 min. MS (ESI)$^-$: *m/z* 581.8 [M − H]$^-$. UV vis λ_{max} = 527 nm.

4.2.3.19. (E)-5-Ammonio-3-(2-(2-fluoro-2-iodophenyl)hydrazono)-4-oxo-3,4-dihydronaphthalene-2,7-disulfonate ***4l*** *(LSP9-2126).* Following the general procedure B, corresponding diazonium salt (2.5 mmol) was added to 5-Amino-4-hydroxynaphthalene-2,7-disulfonic acid (445 mg, 1.3 mmol). The crude product was

purified by preparative HPLC gradient P2. Compound was the first eluted and obtained as a violet powder in 3% yield (25 mg). ^{1}H NMR (500 MHz, DMSO): δ 15.36 (s, 1H, NH); 7.76 (d, J = 8.5 Hz, 1H, $H_{6'}$); 7.75 (dd, J = 10.3 Hz, J = 1.7 Hz, 1H, $H_{3'}$); 7.64 (dd, J = 8.5 Hz, J = 1.7 Hz, 1H, $H_{5'}$); 7.29 (s, 1H, H_1); 7.08 (d, J = 1.3 Hz, 1H, H_6); 6.84 (d, J = 1.3 Hz, 1H, H_8). ^{13}C NMR (126 MHz, DMSO): δ 180.8 (C_4); 156.8 (C_7); 154.6 (C_2); 150.7 ($C_{3'}$); 146.3 ($C_{5'}$); 145.4 (C_5); 140.5 ($C_{6'}$); 134.6 (C_9); 134.1 ($C_{1'}$); 130.5 (C_3); 124.7 (C_1); 123.5 ($C_{2'}$); 117.2 (C_8); 115.1 (C_6); 111.2 (C_{10}); 89.3 ($C_{4'}$). Elemental analysis found (calc) (+NaCl; +3H_3O^+): C 28.61 (28.93); H 2.58 (2.66); N 6.09 (6.10). HPLC (Gradient A) t_R = 10.61 min. MS (ESI)$^-$: m/z 565.9 [M − H]$^-$. UV vis λ_{max} = 536 nm.

Compound **16l** was the second eluted in the purification of **4l** and obtained as a blue powder in 5% yield (42 mg). ^{1}H NMR (500 MHz, DMSO): δ 10.53 (s, 1H, NH); 10.30 (s, 1H, NH); 7.90 (dd, J = 10 Hz, J = 1.6 Hz, 1H, $H_{3''}$); 7.85 (dd, J = 10.4 Hz, J = 1.4 Hz, 1H, $H_{3'}$); 7.81 (t, J = 8.4 Hz, 1H, $H_{6''}$); 7.80 (t, J = 8.3 Hz, 1H, $H_{6'}$); 7.73 (dd, J = 8.6 Hz, J = 1.4 Hz, 1H, $H_{5''}$); 7.70 (dd, J = 8.6 Hz, J = 1.4 Hz, 1H, $H_{5'}$); 7.47 (s, 1H, H_6); 7.41 (s, 1H, H_8). ^{13}C NMR (126 MHz, DMSO): δ 180.7; 169.2; 154.7; 146.4; 145.3; 140.6; 140.3; 134.5; 134.0; 130.5; 129.0; 125.4; 124.5; 123.4; 122.5; 120.0; 119.1; 117.1; 115.0; 109.7; 96.2 HPLC (Gradient A) t_R = 14.11 min. MS (ESI)$^-$: m/z 813.8 [M − H]$^-$. UV vis λ_{max} = 600 nm.

4.2.3.20. (E)-5-Ammonio-3-(2-(4-iodo-2-(trifluoromethyl)phenyl) hydrazono)-4-oxo-3,4-dihydronaphthalene-2,7-disulfonate, ***4m*** *(LSP9-2119).* Following the general procedure B, corresponding diazonium salt (3.7 mmol) was added to 5-Amino-4-hydroxynaphthalene-2,7-disulfonic acid (1130 mg, 3.3 mmol). The crude product was purified by preparative HPLC gradient P2. Compound was the first eluted and obtained as a dark blue powder in 2% yield (40 mg). ^{1}H NMR (500 MHz, DMSO): δ 11.05 (brs, 1H, NH); 8.00 (m, 3H, $H_{3'}$, $H_{5'}$, $H_{6'}$); 7.43 (s, 1H, H_1); 7.18 (s, 1H, H_6); 7.00 (s, 1H, H_8). ^{13}C NMR (126 MHz, DMSO): δ 172.3 (C_4); 150.3 (C_7); 150.2 (C_2); 145.2 ($C_{5'}$); 143.8 ($C_{1'}$); 142.1 (C_5); 136.2 (C_9); 134.1 ($C_{3'}$); 125.8 (C_3); 125.1 ($C_{6'}$–$C_{7'}$); 120.3 (C_1); 117.2 (C_8); 115.2 ($C_{2'}$); 113.6 (C_6); 110.1 (C_{10}); 91.5 ($C_{4'}$). HPLC (Gradient A) t_R = 12.38 min. MS (ESI)$^-$: m/z 615.9 [M − H]$^-$. UV vis λ_{max} = 521 nm.

4.2.3.21. (E)-5-Ammonio-3-(2-(4-bromo-2,3,5-trifluoro-6-hydroxyphenyl)hydrazono)-4-oxo-3,4-dihydronaphthalene-2,7-disulfonate, ***4n*** *(LSP9-3032).* Following the general procedure B, corresponding diazonium salt (1.4 mmol) was added to 5-Amino-4-hydroxynaphthalene-2,7-disulfonic acid (410 mg, 1.2 mmol) in 10 mL of H_2O. The crude product was purified by preparative HPLC using gradient P2. Compound was obtained as a dark blue solid in 74% yield (510 mg). ^{1}H NMR (500 MHz, DMSO): δ 15.55 (s, 1H, NH); 10.72 (s, 1H, OH); 7.28 (s, 1H, H_1); 7.14 (s, 1H, H_6); 6.9 (s, 1H, H_8). ^{13}C NMR (126 MHz, DMSO): δ 182.4 (C_4); 154.5 (C_7); 154.1 (C_2); 145.5 ($C_{5'}$); 140.8 (C_5); 140.1 ($C_{3'}$); 136.5 ($C_{2'}$); 135.9 (C_9); 134.4 ($C_{6'}$); 129.8 (C_3); 124.6 (C_1); 119.0 ($C_{1'}$); 114.7 (C_8); 113.7 (C_6); 111.0 (C_{10}); 92.54 ($C_{4'}$). ^{19}F NMR (376 MHz, DMSO): −133.90 (s, 1F, $F_{5'}$); −145.20 (d, J = 28 Hz, 1F, $F_{2'}$); −157.93 (d, J = 28 Hz, 1F, $F_{3'}$). Elemental analysis found (calcd) (+Na^+; +2 Cl^-; +2H_3O^+; +H_2O): C 26.57 (26.57); H 2.20 (2.09); N 5.46 (5.81). HPLC (Gradient A) t_R = 12.30 min. MS (ESI)$^-$: m/z 569.9 [M − H]$^-$. UV vis λ_{max} = 539 nm.

4.2.3.22. (E)-5-Ammonio-3-(2-(4-bromo-2-methoxyphenyl)hydrazono)-4-oxo-3,4-dihydronaphthalene-2,7-disulfonate, ***4o*** *(LSP9-2122).* Following the general procedure B, corresponding diazonium salt (2.4 mmol) was added to 5-Amino-4-hydroxynaphthalene-2,7-disulfonic acid monosodium salt (716 mg, 2.1 mmol). Compound was obtained as a violet powder in 20% yield (245 mg). ^{1}H NMR (500 MHz, DMSO): δ 15.35 (s, 1H, NH); 7.85 (d, J = 8.6 Hz, 1H, $H_{6'}$); 7.33 (d, J = 1.7 Hz, 1H, $H_{3'}$); 7.29 (s, 1H, H_1); 7.24 (dd, J = 8.6 Hz, J = 1.7 Hz, 1H, $H_{5'}$); 7.06 (s, 1H, H_8); 6.87 (s, 1H, H_6); 4.00 (s, 3H, $H_{7'}$). ^{13}C NMR (126 MHz, DMSO): δ 181.4 (C_4); 153.5 (C_7); 153.1 (C_2); 148.1 ($C_{2'}$); 142.8 (C_5); 136.3 (C_9); 131.1 ($C_{1'}$); 129.9 (C_3); 124.1 ($C_{5'}$); 123.0 (C_1); 116.9 ($C_{6'}$); 116.3 ($C_{4'}$); 114.6 ($C_{3'}$); 113.5 (C_8); 112.1 (C_6); 111.8 (C_{10}); 56.6 ($C_{7'}$). Elemental analysis found (calcd.) (+NaCl; +3H_3O^+): C 30.48 (30.56); H 3.31 (3.29); N 6.27 (6.12); S 9.57 (9.41). HPLC (Gradient A) t_R = 10.53 min. MS (ESI)$^-$: m/z 530.1 [M − H]$^-$. UV vis λ_{max} = 543 nm.

4.2.3.23. (E)-5-Ammonio-3-(2-(4-bromo-2,6-dimethylphenyl)hydrazono)-4-oxo-3,4-dihydronaphthalene-2,7-disulfonate, ***4p*** *(LSP9-2125).* Following the general procedure B, corresponding diazonium salt (1.4 mmol) was added to 5-Amino-4-hydroxynaphthalene-2,7-disulfonic acid monosodium salt (445 mg, 1.3 mmol). Compound was obtained as a purple powder in 24% yield (180 mg). ^{1}H NMR (500 MHz, DMSO): δ 16.31 (s, 1H, NH); 7.33 (s, 2H, $H_{3'}$, $H_{5'}$); 7.31 (s, 1H, H_1); 7.04 (s, 1H, H_6); 6.87 (s, 1H, H_8); 2.55 (s, 6H, $H_{7'}$). ^{13}C NMR (126 MHz, DMSO): δ 181.0 (C_4); 153.5 (C_7); 153.0 (C_2); 142.7 (C_5); 138.0 ($C_{1'}$); 136.9 (C_9); 131.6 ($C_{3'}$–$C_{5'}$); 129.8 (C_3); 122.3 (C_1); 116.7 ($C_{2'}$–$C_{6'}$); 113.1 (C_8); 112.1 (C_6); 111.6 (C_{10}); 106.8 ($C_{4'}$); 19.5 ($C_{7'}$). Elemental analysis found (calcd.) (+NaCl; +2H_3O^+): C 33.49 (33.42); H 3.47 (3.26); N 6.16 (6.48); S 9.76 (9.88). HPLC (Gradient A) t_R = 11.96 min. MS (ESI)$^-$: m/z 530.0 [M − H]$^-$. UV vis λ_{max} = 523 nm.

4.2.3.24. ***5d*** *LSP9-3024 and* ***5b*** *-LSP9-3088.* NaOH (20 g, 0.5 mol) was dissolved in 60 mL of water. 4-Bromo-2-fluoroaniline (19 g, 0.1 mol), sodium formate (6.8 g, 0.1 mol), cetyl trimethylammonium bromide (CTAB) (4 g, 0.01 mol) and palladium on activated charcoal (2 g) were added to the solution. The reaction mixture was stirred under reflux for 4 h. Afterwards, another batch of sodium acetate (6.8 g, 0.1 mol), was added to the solution. The reaction mixture was heated under reflux for another 20 h. After cooling down to room temperature, the reaction mixture was filtered and the residual solid washed with EtOAc (500 mL). This layer was filtered to remove salts. Solvents were removed under reduced pressure. The crude product was purified by column chromatography on silica gel (Cyclohexane/EtOAc: 70/30). The first eluted product was 3,3′-difluorobiphenyl-4,4′-diamine (**5d**), followed by 2-fluorobenzidine and benzidine **5b**).

3,3′-Difluoro-[1,1′-biphenyl]-4,4′-diamine **5d** was obtained as an orange powder in 24% yield (2.62 g) ^{1}H NMR (500 MHz, CD_3CN): δ 7.20 (dd, J = 13 Hz, J = 2 Hz, 2H, H_2, $H_{2'}$); 7.14 (dd, J = 8.2 Hz, J = 2 Hz, 2H, H_6, $H_{6'}$); 6.82 (t, J = 8.8 Hz, 2H, H_5, $H_{5'}$); 4.19 (brs, 4H, 2 × NH_2). ^{13}C NMR (126 MHz, CD_3CN): δ 152.7 (d, J = 236.8 Hz, C_3, $C_{3'}$); 135.5 (d, J = 13.4 Hz, C_4, $C_{4'}$); 130.9 (d, J = 6.3 Hz, C_1, $C_{1'}$); 123.1 (d, J = 2.4 Hz, C_6, $C_{6'}$); 117.9 (d, J = 4.4 Hz, C_5, $C_{5'}$); 113.6 (d, J = 19.5 Hz, C_2, $C_{2'}$).

Benzidine **5b** was obtained as an orange powder in 10% yield (1.12 g) ^{1}H NMR (500 MHz, CD_3CN): δ 7.31 (d, J = 8.2 Hz, 4H, H_2, H_6, $H_{2'}$, $H_{6'}$); 6.62 (d, J = 8.2 Hz, 4H, H_3, H_5, $H_{3'}$, $H_{5'}$); 4.09 (brs, 4H, 2 × NH_2).

4.2.3.25. 3,3′-Dimethyl-[1,1′-biphenyl]-4,4′-bis(diazonium), ***6a***. Following the general procedure A, tolidine (637 mg, 3 mmol), HCl 37% (1 mL, 12 mmol), $NaNO_2$ in solid (166 mg, 2.4 mmol), solvent H_2O/EtOH. The crude product was concentrated in vacuo then extracted by adding 200 mL of HCL 1 M solution to pH = 2 and EtOAc. Compound was obtained as an orange powder in 41% yield (300 mg). ^{1}H NMR (500 MHz, MeOD): δ 8.59 (d, J = 8.8 Hz, 1H, $H_{6'}$); 8.15 (d, J = 1.2 Hz, 1H, $H_{3'}$); 8.07 (dd, J = 8.8 Hz, J = 1.2 Hz, 1H, $H_{5'}$); 7.84 (d, J = 1.8 Hz, 1H, $H_{3''}$); 7.79 (dd, J = 8.2 Hz, J = 1.8 Hz, 1H, $H_{5''}$); 7.37 (d, J = 8.2 Hz, 1H, $H_{6''}$); 2.85 (s, 3H, $H_{7'}$); 2.45 (s, 3H, $H_{7''}$).

4.2.3.26. 4′-Ammonio-3,3′-dichloro-[1,1′-biphenyl]-4-diazonium, ***6c***. Following the general procedure A, 3,3′-Dichlorobenzidine (489 mg, 1.5 mmol), HCl 37% (0.5 mL, 6 mmol), $NaNO_2$ in solid (62 mg, 0.9 mmol), solvent H_2O. The crude product was concentrated in vacuo then extracted by adding 100 mL of HCL 1 M solution to pH = 2 and EtOAc. The aqueous layer was evaporated. Compound was obtained as a brown-orange powder in 45% yield (140 mg). ^{1}H NMR (500 MHz, MeOD): δ 8.55 (d, J = 8.2 Hz, 1H, $H_{6'}$); 8.26 (s, 1H, $H_{3'}$); 8.05 (d, J = 8.2 Hz, 1H, $H_{5'}$); 7.93 (d, J = 1.3 Hz, 1H, $H_{3''}$); 7.70 (dd, J = 8.1 Hz, J = 1.3 Hz, 1H, $H_{5''}$); 7.08 (d, J = 8.1 Hz, 1H, $H_{6'}$).

4.2.3.27. 4′-Ammonio-3,3′-difluoro-[1,1′-biphenyl]-4-diazonium, ***6d***. Following the general procedure A, LSP9-3024 (880 mg, 4 mmol), HCl 37% (1.33 mL, 16 mmol), $NaNO_2$ in solution (3.6 mL, 3.6 mmol), solvent EtOH. The crude product was concentrated in vacuo then extracted by adding 100 mL of HCL 1 M solution to pH = 2 and EtOAc. The aqueous layer was evaporated. Compound was obtained as an orange powder in 50% yield (545 mg). ^{1}H NMR (500 MHz, MeOD): δ 8.55 (dd, J = 9.1 Hz, J = 6.5 Hz 1H, $H_{5'}$); 8.15 (dd, J = 11.9 Hz, J = 1.7 Hz, 1H, $H_{3'}$); 8.04 (dd, J = 9.1 Hz, J = 1.7 Hz, 1H, $H_{6'}$); 7.72 (m, 2H, $H_{3''}$–$H_{5''}$); 7.11 (t, J = 8.7 Hz, 1H, $H_{6''}$).

4.2.3.28. (E)-5-Ammonio-3-(2-(4′-ammonio-3,3′-dimethyl-[1,1′-biphenyl]-4-yl)hydrazono)-4-oxo-3,4-dihydronaphthalene-2,7-disulfonate, ***7a*** *(LSP9-2187).* Following the general procedure C, corresponding diazonium salt (0.8 mmol) was added to 5-Amino-4-hydroxynaphthalene-2,7-disulfonic acid monosodium salt (205 mg, 0.6 mmol). Compound was obtained as a red powder in 25% yield (82 mg). ^{1}H NMR (500 MHz, DMSO): δ 15.82 (s, 1H, NH); 8.02 (d, J = 8.4 Hz, 1H, $H_{6''}$); 7.60 (s, 1H, $H_{3''}$); 7.55 (m, 3H, $H_{3'}$, $H_{6'}$, $H_{5''}$); 7.32 (s, 1H, H_1); 7.31 (d, J = 8.2 Hz, 1H, $H_{5'}$); 7.06 (s, 1H, H_6); 6.88 (s, 1H, H_8); 2.47 (s, 3H, $H_{7''}$); 2.36 (s, 3H, $H_{7'}$). ^{13}C NMR (126 MHz, DMSO): δ 181.2; 153.3; 153.1; 142.7; 140.2; 136.3; 134.7; 133.4; 129.8; 128.9; 128.5; 125.3; 125.0; 124.6; 122.7; 122.7; 122.6; 122.6; 115.9; 113.4; 112.0; 111.9. Elemental analysis found (calcd) (+NaCl; $+H_3O^+$; $+2H_2O$): C 42.00 (41.98); H 4.29 (4.29); N 7.75 (8.10); S 8.68 (9.27). HPLC (Gradient A): t_R = 9.95 min. MS (ESI)$^-$: m/z 541.1 [M − H]$^-$. UV vis λ_{max} = 545 nm.

4.2.3.29. (E)-5-Ammonio-3-(2-(4′-ammonio-[1,1′-biphenyl]-4-yl) hydrazono)-4-oxo-3,4-dihydronaphthalene-2,7-disulfonate, ***7b*** *(LSP9-2142).* Following the general procedure C, corresponding diazonium salt (1.5 mmol) was mixed with 5-Amino-4-hydroxynaphthalene-2,7-disulfonic acid monosodium salt (308 mg, 0.9 mmol). Compound was obtained as a dark blue powder in 40% yield (186 mg). ^{1}H NMR (500 MHz, DMSO): δ 15.24 (s, 1H, NH); 7.70 (m, 6H, $H_{2''}$–$H_{3''}$–$H_{5''}$–$H_{6''}$–$H_{3'}$–$H_{5'}$); 7.36 (d, J = 8.6 Hz, 2H, $H_{2'}$–$H_{6'}$); 7.30 (s, 1H, H_1); 7.06 (s, 1H, H_6); 6.88 (s, 1H, H_8). ^{13}C NMR (126 MHz, DMSO): δ 181.3; 153.5; 153.0; 142.6; 142.30; 135.1; 134.6; 129.1; 127.3; 127.3; 122.8; 116.8; 113.4; 112.0; 111.9. Elemental analysis found (calcd) ($+Na^+$; $+3H_3O^+$): C 44.18 (44.44); H 4.31 (4.58); N 9.06 (9.42); S 10.09 (10.79). HPLC (Gradient A): t_R = 8.44 min. MS (ESI)$^-$: m/z 513.1 [M−H]$^-$. UV vis λ_{max} = 537 nm.

4.2.3.30. (E)-5-Ammonio-3-(2-(4′-ammonio-3,3′-dichloro-[1,1′-biphenyl]-4-yl)hydrazono)-4-oxo-3,4-dihydronaphthalene-2,7-disulfonate, ***7c*** *(LSP9-3075).* Following the general procedure C, corresponding diazonium salt (3 mmol) was mixed with 5-Amino-4-hydroxynaphthalene-2,7-disulfonic acid monosodium salt (285 mg, 0.9 mmol). Compound was obtained as a dark blue powder in 42% yield (220 mg). ^{1}H NMR (500 MHz, DMSO): δ 15.69 (s, 1H, NH); 8.10 (d, J = 8.7 Hz, 1H, $H_{6'}$); 7.76 (d, J = 1.3 Hz, 1H, $H_{3'}$); 7.69 (dd, J = 8.7 Hz, J = 1.3 Hz, 1H, $H_{5'}$); 7.64 (d, J = 1.9 Hz, 1H, $H_{3''}$); 7.47 (dd, J = 8.2 Hz, J = 1.9 Hz, 1H, $H_{5''}$); 7.32 (s, 1H, H_1); 7.08 (s, 1H, H_6); 6.91 (d, J = 8.2 Hz, 1H, $H_{6''}$); 6.88 (s, 1H, H_8). ^{13}C NMR (126 MHz, DMSO): δ 181.5; 153.8; 153.3; 142.7; 137.5; 136.20; 135.9; 130.4; 126.7; 125.8; 125.5; 123.5; 120.4; 117.5; 113.8; 113.7; 112.4; 11.5. Elemental analysis found (calcd) (+NaCl; $+H_3O^+$; $+2H_2O$): C 40.18 (40.48); H 3.72 (3.69); N 8.15 (8.21); S 8.68 (9.40). HPLC (Gradient A): t_R = 12.71 min. MS (ESI)$^-$: m/z 583.01[M−H]$^-$. UV vis λ_{max} = 551 nm.

4.2.3.31. (E)-5-ammonio-3-(2-(4′-ammonio-3,3′-difluoro-[1,1′-biphenyl]-4-yl)hydrazono)-4-oxo-3,4-dihydronaphthalene-2,7-disulfonate, ***7d*** *(LSP9-3073).* Following the general procedure C, corresponding diazonium salt (4 mmol) was mixed 5-Amino-4-hydroxynaphthalene-2,7-disulfonic acid monosodium salt (445 mg, 0.7 mmol). The crude product was purified by preparative HPLC gradient P2. Compound was obtained as a dark blue powder in 41% yield (159 mg). ^{1}H NMR (500 MHz, DMSO): δ 15.63 (s, 1H, NH); 8.02 (t, J = 8.6 Hz, 1H, $H_{6''}$); 7.63 (dd, J = 13.1 Hz, J = 1.4 Hz, 1H, $H_{3''}$); 7.58 (dd, J = 8.6 Hz, J = 1.4 Hz, 1H, $H_{5''}$); 7.50 (dd, J = 12.7 Hz, J = 1.7 Hz, 1H, $H_{3'}$); 7.37 (dd, J = 8.6 Hz, J = 1.7 Hz, 1H, $H_{5'}$); 7.31 (s, 1H, H_1); 7.08 (s, 1H, H_6); 6.90 (t, J = 8.6 Hz, 1H, $H_{6'}$); 6.87 (s, 1H, H_8). ^{13}C NMR (126 MHz, DMSO): δ 181.6 (C_4); 153.7 (C_7); 153.3 (C_2); 152.2 ($C_{2''}$); 150.3 ($C_{2'}$); 142.6 (C_5); 139.2 ($C_{4''}$); 136.2 (C_9); 136.0 ($C_{4'}$); 130.3 (C_3); 129.0 ($C_{1'}$); 123.3 (C_1); 122.5 ($C_{5''}$); 122.4 ($C_{5'}$); 117.4 ($C_{6''}$); 117.2 ($C_{6'}$); 113.6 (C_8); 113.0 ($C_{3'}$); 112.5 ($C_{1''}$); 112.3 (C_6); 112.2 ($C_{3''}$); 111.6 (C_{10}). Elemental analysis found (calcd) ($+NH_4Cl + H_3O^+Cl^- + 3H_2O$): C 37.19 (37.08); H 4.12 (4.10); N 9.85 (9.83); S 8.65 (9.00). HPLC (Gradient A): t_R = 10.63 min. MS (ESI)$^-$: m/z 549.2 [M−H]$^-$. UV vis λ_{max} = 548 nm.

4.2.3.32. (E)-5-Ammonio-3-(2-(4′-ammonio-3,3′-bis(trifluoromethyl)-[1,1′-biphenyl]-4-yl)hydrazono)-4-oxo-3,4-dihydronaphthalene-2,7-disulfonate, ***7e*** *(LSP9-3070).* Following the general procedure C, corresponding diazonium salt (2 mmol) was mixed with 5-Amino-4-hydroxynaphthalene-2,7-disulfonic acid monosodium salt (223 mg, 0.7 mmol). The crude product was purified twice by preparative HPLC using gradient P2 then gradient P1. Compound was obtained as a dark blue powder in 2% yield (11 mg). ^{1}H NMR (500 MHz, DMSO): δ 15.69 (s, 1H, NH); 8.10 (m, 6H, $H_{3'}$–$H_{5'}$–$H_{6'}$–$H_{3''}$–$H_{5''}$–$H_{6''}$); 7.35 (s, 1H, H_1); 7.12 (s, 1H, H_6); 6.89 (s, 1H, H_8). ^{13}C NMR (126 MHz, DMSO): δ 182.0; 172.0; 171.4; 162.8; 154.2; 153.8; 150.2; 145.7; 142.6; 139.9; 136.2; 136.1; 133.6; 132.3; 131.2; 126.0; 125.3; 124.6; 119.0; 118.1; 114.1; 113.4; 113.0; 111.1. Elemental analysis found (calcd) ($+0.5\ (NH_4)_2SO_4 + NH_4Cl + 4H_2O$): C 32.93 (32.98); H 3.92 (3.92); N 10.21 (10.42); S 9.15 (9.17). HPLC (Gradient A): t_R = 11.042 min. MS (ESI)$^-$: m/z 649.6 [M−H]$^-$. UV vis λ_{max} = 566 nm.

4.2.3.33. (E)-5-Ammonio-3-(2-(4′-ammonio-2,2′,3,3′,5,5′,6,6′-octafluoro-[1,1′-biphenyl]-4-yl)hydrazono)-4-oxo-3,4-dihydronaphthalene-2,7-disulfonate, ***7f*** *(LSP9-3071).* Following the general procedure C, corresponding diazonium salt (2 mmol) was mixed 5-Amino-4-hydroxynaphthalene-2,7-disulfonic acid monosodium salt (223 mg, 0.7 mmol). The crude product was purified by preparative HPLC gradient P2. Compound was obtained as a dark blue powder in 2% yield (9 mg). ^{1}H NMR (500 MHz, DMSO): δ 16.12 (s, 1H, NH); 7.29 (s, 1H, H_1); 7.11 (s, 1H, H_6); 6.87 (s, 1H, H_8). ^{13}C N MR (126 MHz, DMSO): δ 183.6; 155.6; 155.2; 154.2; 152.3; 152.3; 144.6; 138.2; 138.0; 138.0; 132.3; 131.0; 131.0; 125.3; 124.5; 124.4; 119.3; 115.6; 115.0; 114.8; 114.3; 114.2; 113.6. HPLC (Gradient A): t_R = 11.709 min. MS (ESI)$^-$: m/z 680.6 [M−H + Na+]$^-$. UV vis λ_{max} = 568 nm.

4.2.3.34. (Z)-4-(2-(2-Chloro-4-iodophenyl)hydrazono)-3-oxo-3,4-dihydronaphthalene-2,7-disulfonate, ***9***. Following the general

procedure A, diazonium of 2-Chloro-4-iodoaniline (508 mg, 2 mmol), was formed and added to 2-Naphtol-3,6-disulfonic acid (488 mg, 1.4 mmol) following Procedure B. Compound was obtained as an orange-red powder in 75% yield (594 mg). ^{1}H NMR (500 MHz, DMSO): δ 16.40 (s, 1H, NH); 8.42 (d, J = 8.2 Hz, 1H, H_5); 8.31 (s, 1H, H_1); 8.04 (s, 1H, $H_{3'}$); 7.98 (d, J = 8.4 Hz, 1H, $H_{5'}$); 7.96 (s, 1H, H_8); 7.86 (d, J = 8.4 Hz, 1H, $H_{6'}$); 7.83 (d, J = 8.4 Hz, 1H, H_6) ^{13}C NMR (126 MHz, DMSO): δ 172.9 (C_4); 147.1 (C_7); 141.1 (C_2); 139.6 ($C_{1'}$); 139.5 (C_1); 137.5 ($C_{3'}$, $C_{6'}$); 132.8 (C_9); 131.1 (C_4); 127.5 (C_6); 126.6 (C_8); 125.92 (C_{10}); 123.8 ($C_{2'}$); 121.5 (C_5); 118.7 ($C_{5'}$); 91.2 ($C_{4'}$). HPLC (Gradient A): t_R = 9.87 min. MS (ESI)$^+$: m/z 567.1 $[M+H]^+$. UV vis λ_{max} = 493 nm.

4.2.3.35. (E)-3-(2-(2-Chloro-4-iodophenyl)hydrazono)-4-oxo-3,4-dihydronaphthalene-2,7-disulfonate, ***11****.* Following the general procedure A, 2-Chloro-4-iodoaniline (508 mg, 2 mmol), HCl 37% (0.7 mL, 8 mmol), $NaNO_2$ in solid (180 mg, 2.6 mmol), solvent H_2O. 1-naphtol-3,6-disulfonic acid (455 mg, 1.4 mmol). Compound was obtained as a red powder in 40% yield (321 mg). ^{1}H NMR (500 MHz, DMSO): δ 16.12 (s, 1H, NH); 8.26 (d, J = 8.2 Hz, 1H, H_5); 8.00 (d, J = 1.4 Hz, 1H, $H_{3'}$); 7.89 (d, J = 8.7 Hz, 1H, $H_{6'}$); 7.88 (dd, J = 8.7 Hz, J = 1.4 Hz, 1H, $H_{5'}$); 7.87 (d, J = 1.6 Hz, 1H, H_8); 7.75 (dd, J = 8.2 Hz, J = 1.6 Hz, 1H, H_6); 7.59 (s, 1H, H_1). ^{13}C NMR (126 MHz, DMSO): δ 176.9 (C_4); 152.9 (C_7); 143.5 (C_2); 139.2 ($C_{2'}$); 137.4 ($C_{6'}$); 137.2 ($C_{3'}$); 135.4 (C_{10}); 130.3 (C_3); 129.8 (C_9); 126.9 (C_5); 125.5 (C_8); 125.1 (C_6); 122.3 ($C_{1'}$); 121.9 (C_1); 119.3 ($C_{5'}$); 90.2 ($C_{4'}$). HPLC (Gradient A): t_R = 10.53 min. MS (ESI)$^+$: m/z 567.1 $[M+H]^+$. UV vis λ_{max} = 500 nm.

4.2.3.36. (3E,8Z)-3,8-Bis(2-(2-chloro-4-iodophenyl)hydrazono)-7-imino-4-oxo-3,4,7,8-tetrahydronaphthalene-2-sulfonate, ***13****.* Following the general procedure B, corresponding diazonium salt (1.4 mmol) was added to 7-Amino-4-hydroxy-2-naphatlendisulfonic acid (445 mg, 1.3 mmol). Compound was obtained as a dark blue powder in 42% (345 mg). ^{1}H NMR (500 MHz, DMSO): δ 16.36 (s, 1H, NH); 9.96 (s, 1H, NH); 8.65 (s, 1H, H_1); 8.14 (d, J = 8.9 Hz, 1H, H_5); 8.12 (d, J = 1.8 Hz, 1H, $H_{3''}$); 7.97 (d, J = 1.3 Hz, 1H, $H_{3'}$); 7.94 (dd, J = 8.3 Hz, J = 1.8 Hz, 1H, $H_{5''}$); 7.84 (m, 2H, $H_{5'}$–$H_{6'}$); 7.59 (d, J = 8.3 Hz, 1H, $H_{6'}$); 7.05 (d, J = 8.9 Hz, 1H, H_6). ^{13}C NMR (126 MHz, DMSO): δ 174.6 (C_4); 147.6 ($C_{2''}$); 146.2 (C_7); 144.8 (C_2); 139.6 ($C_{2'}$); 139.2 (C_8); 138.2 ($C_{3''}$); 137.2 ($C_{3'}$, $C_{5'}$, $C_{5''}$); 133.4 ($C_{1''}$); 131.7 (C_5); 130.1 (C_3); 128.6 (C_{10}); 122.4 ($C_{1'}$); 120.0 (C_9); 119.1 ($C_{6'}$); 118.2 (C_6); 117.7 ($C_{6''}$); 117.2 (C_1); 96.9 ($C_{4''}$); 89.8 ($C_{4''}$). HPLC (Gradient A) t_R = 20.61 min. MS (ESI)$^-$: m/z 766.2 $[M-H]^-$.

4.3. Vesicular neurotransmitter uptake transport assay

The Vesicular Glutamate uptake was assayed by slight modification of the method of Naito and Ueda [7] as described by Kish and Ueda [27]. Aliquots (50 μg) of crude rat synaptic vesicles were incubated for 10 min at 37 °C in a solution containing 10 mM HEPES–KOH, pH 7.4, 0.32 mM sucrose, 4 mM MgSO4, 4 mM KCl, 1 mM L-aspartic acid, 100 μM Glu, 2 mM ATP, and 2 μCi [3H]Glu (Perkin Elmer) in a final volume of 0.1 mL. Test compounds were dissolved in DMSO and added in 0.6 μL aliquots to give final concentrations between 0.20 nM and 200 μM. Uptake was initiated by addition of a mixture A of 2 mM ATP and 100 μM Glu (final concentration) containing 2 μCi of [3H]Glu. After 10 min at 37 °C, 2.5 ml of icecold uptake buffer were added and the mixture immediately filtered through Whatman GF/C glass-fiber filter paper (25 mm) using a manifold under vacuum. The filters were washed 4 times with 2.5 mL of uptake buffer and placed in scintillation vials with scintillation cocktail (Cytoscint, ICN). The vials were shaken overnight and radioactivity determined in a Beckman LS 6500 scintillation spectrophotometer. Values obtained in the absence of ATP or by inhibition by CCCP or Trypan Blue were subtracted from those in the presence of ATP to calculate ATP dependent uptake activity. The amount of radioactive Glu trapped on GF/C glass-fiber filter paper was indistinguishable from that trapped on the Millipore HAWP filter (data not shown). Since the rate of filtration with GF/C glass-fiber filter paper is higher this type of filter was used in all experiments described here. Similar uptake protocols were used with other neurotransmitters. Statistical and IC_{50} calculation have been performed using BioDataFit 1.02 software of Chang Bioscience.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.03.056.

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