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Straightforward and effective synthesis of γ-aminobutyric acid transporter subtype 2-selective acyl-substituted azaspiro[4.5] decanes

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ω-Amino acid selectivity

ABSTRACT

Supply of major metabolites such as γ-aminobutyric acid (GABA), β-alanine and taurine is an essential instrument that shapes signalling, proper cell functioning and survival in the brain and peripheral organs. This background motivates the synthesis of novel classes of compounds regulating their selective transport through various fluid-organ barriers via the low-affinity γ-aminobutyric acid (GABA) transporter subtype 2 (GAT2). Natural and synthetic spirocyclic compounds or therapeutics with a range of structures and biological activity are increasingly recognised in this regard. Based on pre-validated GABA transport activity, straightforward and efficient synthesis method was developed to provide an azaspiro[4.5]decane scaffold, holding a variety of charge, substituent and 3D constrain of spirocyclic amine. Investigation of the azaspiro[4.5]decane scaffold in cell lines expressing the four GABA transporter subtypes led to the discovery of a subclass of a GAT2-selective compounds with acyl-substituted azaspiro[4.5]decane core.

Impairment of γ-aminobutyric acid (GABA) signalling has been implicated in disease states associated with asthma, chronic obstructive pulmonary disease and cystic fibrosis or acute lung injury[1,2] suggesting that novel approaches to prevent the loss or even to facilitate GABA signalling are needed. A promising way to achieve this goal is to inhibit GABA transporters and consequently increasing extracellular GABA level.

Localization of the GABA transporter subtype GAT2 (solute carrier family 6 member 13 gene: *slc6a13*; hGAT2, rGAT2, mGAT3) in airway epithelium and smooth muscle[3] suggests a role for GABA signalling. By performing guanidinoacetic acid (GAA) or taurine (Tau) uptake in the sinusoidal membrane of periportal hepatocytes, GAT2 does also regulate creatine or taurocholate biosyntheses in the liver.[4,5] GAT2 in the basolateral membranes of proximal tubules in the renal cortex.[5,6] may possibly be associated with GABA-induced natriuresis and blood pressure reduction. Efflux of Tau from the brain through GAT2 localised in brain blood vessels[5,7] could occur under injurious depolarizing conditions.[8,9] GAT2-mediated uptake of δ-amino levulinic acid (δ-ALA) in the brain leads to accumulation of fluorescent porphyrins in malignant gliomas helping surgery.[10] At present, therefore, the recognition of small molecules regulating GAT2 function in these organs is of great (patho)physiological and pharmacological relevance.

In addition to the presence of GAT2 (*slc6a13)* in *Homo sapiens*, *Rattus norvegicus* (rat) and *Mus musculus* (mouse) mentioned above, similar function also appears in domestic animals such as *Bos taurus* (cow), *Gallus gallus* (chicken), *Apis mellifera* (honeybee) and in model organisms *Danoi rerio* (zebrafish), *Drosophila melanogaster* (fruitfly), *Caenorhabditis elegans*, *Bacillus subtili*, *Halobacterium salinarum*, and in pathogens *Anopheles gambiae*, *Staphylococcus aureus*, *Helicobacter pylori.*[11] Abundance of GAT2 function across organisms signifies the importance of the development of GAT2 subtype-selective inhibitors.

Design and synthesis of a variety of small molecule scaffolds inhibiting GABA transport through the neuronal GAT1 (*slc6a1*; hGAT1, rGAT1, mGAT1) and glial GAT3 (*slc6a11*; hGAT3, rGAT3, mGAT4) subtypes, that control synaptic and extrasynaptic GABA levels, has successfully led to the discovery of potent GAT1- and GAT3-selective inhibitors.[12] These include GAT1-selective conformationally restricted GABA bioisosters[13] Tiagabine, SK&F

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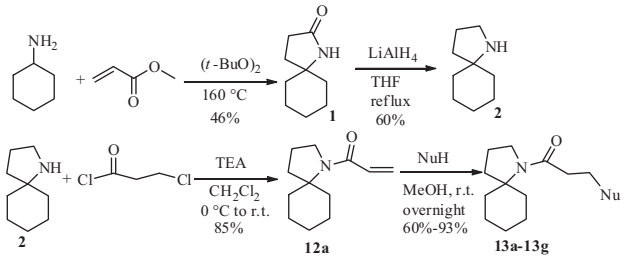
89976-A, CI-966, NNC-711and GAT3-selective non-bioisosteric5-(thiophen-2-yl)indoline-2,3-diones.[14] Conformational restriction of GABA via bicyclo[3.1.0]hexane backbone has provided the most potent and selective inhibitor of the peripheral betain-GABA transporter BGT1 (*slc6a12*; hGAT4, rGAT4, mGAT2).[15]

According to the homology modelling studies of Schlessinger et al. GAT2 is avery selective transporter, compared to other neurotransmitter transporters.[7] Selectivity of GAT2 towards some ω-amino acids such as β-alanine (β-Ala), Tau, 3-amino-1-propanesulfonic acid (homoTau), GAA, δ-ALA, γ-amino-β-(4′-chlorophenil)-butyric acid ((*R*)-baclofen) qualifies GAT2 as a versatile target, possibly retaining distinguishable binding/transport mode of interaction with different substrates/inhibitors. Molecular docking of more than 500,000 compounds along with uptake data of hits concluded that selective GAT2 inhibitors exert their effect at high concentrations (0.5–5 mM), except for the endogenous substrates GABA and β-Ala that are characterised by K_m values of 0.02 mM and 0.03 mM, respectively.[7,16] These findings classify peripheral GAT2 as a selective and low-affinity subtype. Some of the GAT2 inhibitors showed novel scaffolds, rather than those of other GAT transport inhibitors disclosed so far.[7] The similarity of substrate binding crevices of peripheral GAT2 and neuronal GAT3 monomers in the occluded substrate binding conformation,[17] however, suggests that GAT2 versus GAT3 selectivity may perhaps better be achieved by considering the size and the charge-configuration of molecules fitting additional GAT2 conformations as suggested.[7]

Great variety of 3D size and charge-configuration of spirocyclic ring systems may offer a novel scaffold providing GAT2 subtype-selective inhibitors. Living cells synthesize spirocyclic ring systems such as ubiquitous bioactive [5,5]-, [5,6]-, and [6,6]-spiroacetals and alkaloids (for example stepharine, annosqualine, securinine, viroallosecurinine, halichlorine, pinnaic acids, pinnatoxin A, norzoanthamine, histrionicotoxins, rhynchophylline, spirotryprostatin A, erythraline) containing diverse spirocyclic cores.[18,19] The identification of key enzymes dihydroxy ketone and spiroacetal synthases[19] producing reveromycin A has inspired novel biosynthetic routes to get spirocyclic drug leads.[20] Spirocyclic ring systems are increasingly used in drug discovery.[21,22] Leading scaffolds include derivatives of various spirobicyclic amines such *n*-asazaspiro[3.5]nonane (*n* = 5, 6, 7), 7-azaspiro[4.4]nonane (Spiraprilat), *n*-azaspiro[4.5]decane (Buspiron (*n* = 8), Enilospirone (*n* = 4)), 1,3-diazaspiro[4.4]nonane (Irbesartan), 3,8-diazaspiro

Table 1
Synthesis of spiro-bicyclic compounds **13a–13g**



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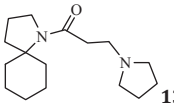
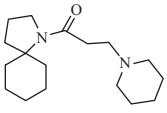
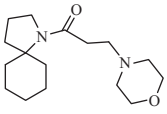
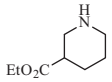
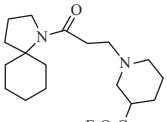
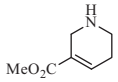
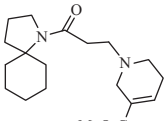
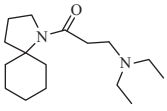
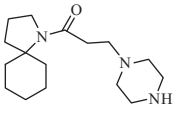
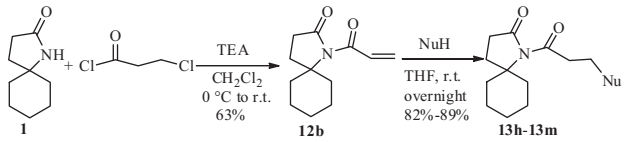
1		 **13a**	93
2		 **13b**	60
3		 **13c**	89
4		 **13d**	76
5		 **13e**	75
6		 **13f**	89
7		 **13g**	79

Table 2
Synthesis of spiro-bicyclic compounds **12b** and **13h–m**



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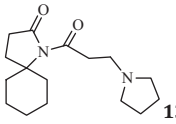
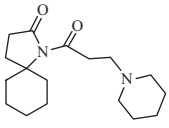
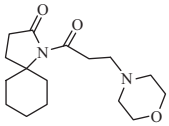
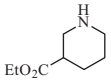
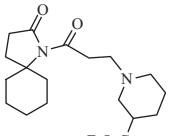
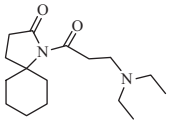
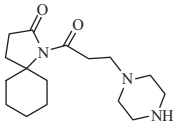
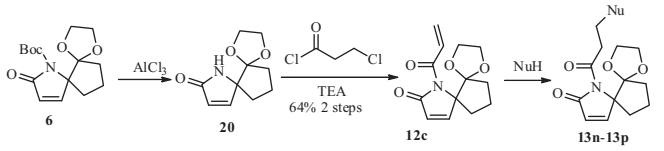
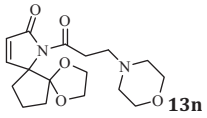
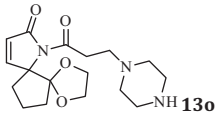
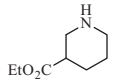
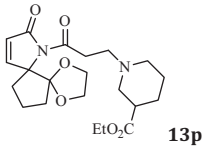
1		 **13h**	89
2		 **13i**	85
3		 **13j**	88
4		 **13k**	83
5		 **13l**	88
6		 **13m**	82

Table 3
Synthesis of spiro-bicyclic compounds **12c** and **13n–p**



Entry	Nu	Product	Yield (%)
1		 **13n**	75
2		 **13o**	78
3		 **13p**	70

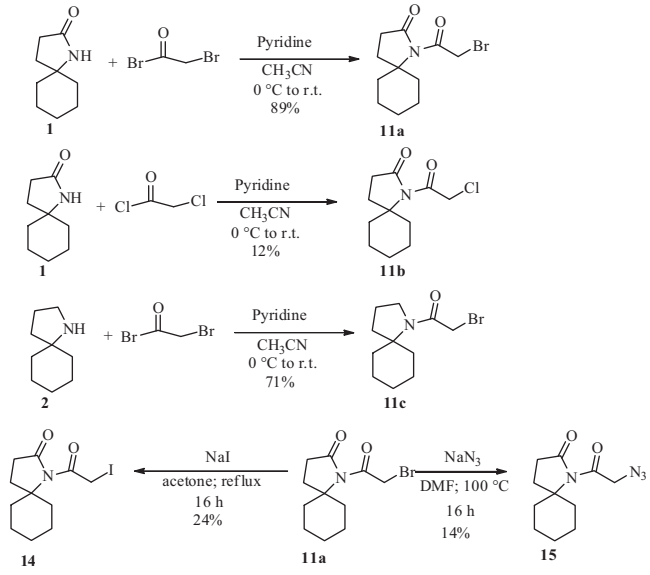


Figure 1. Synthesis of spiro-bicyclic compounds **11a–11c**, **14**and **15**.

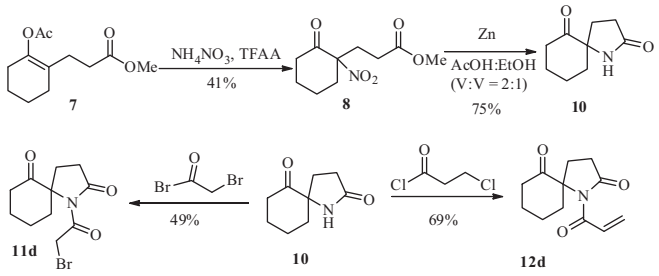


Figure 3. Synthesis of spiro-bicyclic compounds **10**, **11d** and **12d**.

[4.5]decane (Fenspiride), 1,3,8-triazaspiro[4.5]decane (Fluspirilene).[22,23] These developments together with GABA uptake inhibitory activity of 2-azaspiro[4.5]decane-6-carboxylates[24] highlight azaspiro[4.5]decane derivatives as a promising starting scaffold for synthesis and evaluation of GAT subtype selectivity.

The aza-spirobicyclic subunits, core skeletons of the compounds tested during this study have been synthesized according to three different, though complementary routes. Our approach towards the amino-bicycle **2** is based upon the *tert*-butyl peroxide-induced radical cyclization between cyclohexylamine and methyl acrylate to form the spirobicyclic amide **1**[25] which can be further reduced to the desired amine **2** in the presence of $LiAlH_4$ (Table 1). Access to the more substituted bicyclic structures, including the corresponding [4,4,1] spiro derivatives, required a completely different strategy. For that purpose, we set upon a method pioneered in our laboratory and involving the coupling of *tert*-butyl 2-((*tert*-butyldimethylsilyl)oxy)-1*H*-pyrrole-1-carboxylate (TBSOP)[26] **3** with functional orthoester[27] **4** in the presence of $ZnCl_2$ as catalyst. Under these conditions, the iodine-containing bicycle **5** is produced in good yields. Treatment of **5** with *t*-BuOK induces smooth cyclization, affording the desired unsaturated amide **6**. Whilst this approach generates highly functionalized scaffolds, a simpler access to the corresponding [5,4,1] tricycles, such as **10**, was also sought after. Hence, nitration of enol ester **7**[28] provided the α-nitro keto-ester **8** in reasonable yields. Chemoselective reduction of the nitro-group, using zinc in acetic acid, yielded amine **9**[29], which underwent smooth intramolecular cyclization to keto-amide **10**. The spirocyclic scaffolds (**1**, **2**, **6**, **10**), prepared according to these three strategies, can be further transformed, leading to a variety of other spirobicyclic derivatives. For example, reaction of the nitrogen atom with acyl chlorides afforded the corresponding amides **11**, whilst addition of 3-chloro-propionyl chloride produced the unsaturated amides **12**. Finally, Aza-Michael addition between various amines and the unsaturated amides **12** delivered a series of spirobicyclic compounds **13** (Tables 1–3).

The synthesis of compound library containing **1**, **2**, **10**, **11a–11d**, **12a–d**, **13a–p**, **14**, **15**, **18** and **19** is outlined in Tables 2 and 3 and Figs. 1–3. Compounds **1**, **2**, **12a** and **13a–13g** were synthesized according to the pathway described in Table 1. **2** was further

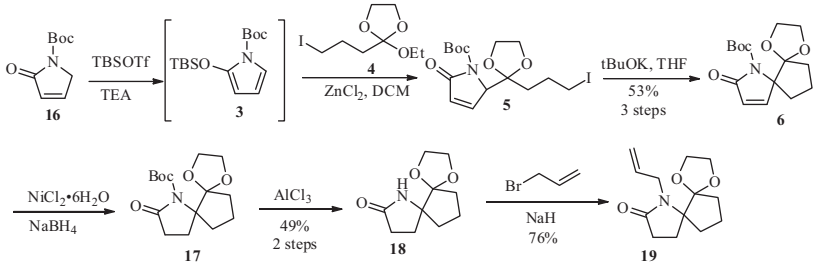


Figure 2. Synthesis of spiro-bicyclic compounds **18** and **19**.

Table 4
Inhibitory effect of test compounds on cloned GAT1, BGT1, GAT2 and GAT3 GABA transporter subtypes[33]

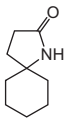
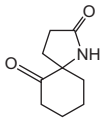
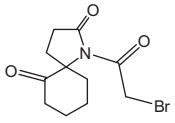
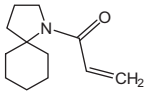
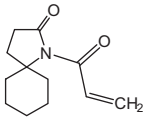
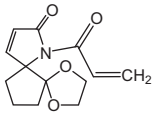
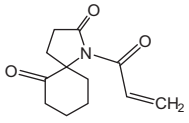
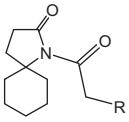
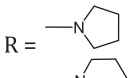
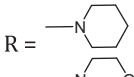
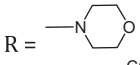
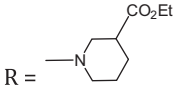
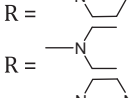
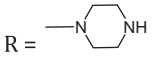
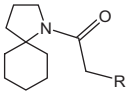
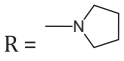
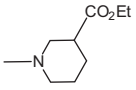
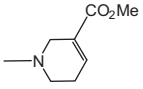
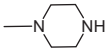
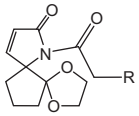
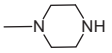
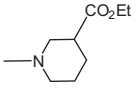
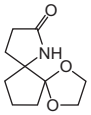
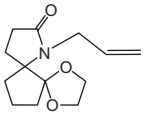
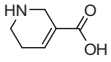
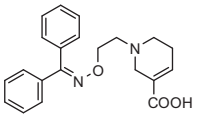
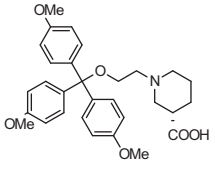
Compounds		IC_{50} (μM, mean ± SEM)*			
		GAT1	BGT1	GAT2	GAT3
1		>5000	>5000	>5000	>5000
2		>5000	>5000	>5000	>5000
10		>1000	>1000	>1000	>1000
11d		>1000	>1000	>1000	>1000
12a		>5000	>5000	3020 ± 457	>5000
12b		371 ± 56	103 ± 23	>1000	>1000
12c		>1000	>1000	>1000	>1000
12d		>1000	>1000	>1000	>1000
					
11a	R = Br	371 ± 56*	103 ± 23*	>1000	>1000
11b	R = Cl	>1000	>1000	>1000	>1000
13h	R = 	>1000	>1000	>1000	>1000
13i	R = 	>1000	>1000	>1000	>1000
13j	R = 	>1000	>1000	>1000	>1000
13k	R = 	>1000	164 ± 15	>1000	933 ± 104
13l	R = 	>1000	>1000	>1000	>1000
13m	R = 	>1000	>1000	>1000	>1000
14	R = I	>1000	>1000	>1000	>1000
15	R = N_3	>1000	>1000	>1000	>1000
					
11c	R = Br	233 ± 13	>5000	>5000	>5000
13a	R = 	>5000	>5000	>5000	675 ± 63

Table 4 (*continued*)

Compounds		IC_{50} (μM, mean ± SEM)*			
		GAT1	BGT1	GAT2	GAT3
13b	R = 	>5000	>5000	>5000	>5000
13c	R = 	>5000	>5000	>5000	>5000
13d	R = 	>5000	>5000	2330 ± 220	>5000
13e	R = 	2050 ± 360	2300 ± 330	3230 ± 280	2860 ± 510
13f	R = 	>1000	>1000	>1000	>1000
13g	R = 	>1000	>1000	>1000	>1000
					
13n	R = 	>1000	>1000	>1000	>1000
13o	R = 	>1000	>1000	>1000	>1000
13p	R = 	>1000	>1000	>1000	>1000
18		>1000	>1000	>1000	>1000
19		>1000	>1000	>1000	>1000
Standards					
21		20.9 ± 1.4	>1000	72.4 ± 4.4	114.8 ± 8.7
22		0.18 ± 0.01	>1000	>1000	>1000
23		199 ± 18	>1000	21.9 ± 1.7	6.3 ± 0.18

* Number of determinations: 3–11; SEM: standard error of mean; >5000 or >1000 denotes measurements in which 50% inhibition were not achieved at the highest tested concentrations (5000 μM or 1000 μM, respectively). Compounds were fully soluble at these concentrations.

reacted with 3-chloro-propionyl chloride, in the presence of triethylamine (TEA), leading directly to the unsaturated amide **12a** in 85% yield. Acrylamide **12a** was then engaged in a series of Michael additions, using a variety of amines, including pyrrolidine, piperidine, morpholine, ethyl piperidine-3-carboxylate, methyl 1,2,5,6-tetrahydropyridine-3-carboxylate, diethylamine, piperazine, providing in all cases, the desired aza-Michael addition products (**13a**–**13g**) in good to excellent yields (Table 1).

Whilst amine **2** can be easily derivatized by a variety of electrophiles, we were also interested in testing the activities of the analogous series bearing an amide function instead of a basic tertiary amine. Hence, amide **1** was thus treated with 3-chloro-propionyl chloride, in the presence of triethylamine affording, as expected, the α,β-unsaturated imide **12b**. Michael addition of a variety of amines gave the desired adducts **13h**–**m** (Table 2).

To diversify even further our small library of aza-spirobicyclic bioisosters of GABA, different alpha-halogenated imides bearing a Cl, Br and I-containing side-chain were synthesized (**11a**–**11c** and **14**). To generate further diversity through click-chemistry, the azide adduct **15** was also designed and synthesized (Fig. 1).

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Having successfully prepared these bicyclo-[5,4,1] derivatives, we turned our attention to the assembly of the analogous spirobicyclic compounds **12c**, **13n**–**13p**, **18** and **19**, bearing a smaller five-membered ring system (Table 3). Reduction of the conjugated double bond with $NaBH_4$ in the presence of $NiCl_2{\cdot}6H_2O$ provided Boc-protected-amide **17**. Finally, deprotection of the Boc group was accomplished by treatment of **17** with a catalytic amount of $AlCl_3$ giving amide **18** in 49% yield over 2 steps.[30] N-allylation of **18**, using allyl bromide and NaH in THF, afforded tertiary amide **19** (Fig. 2).

The Boc-containing compound **6** could also be deprotected using $AlCl_3$, affording the unsaturated amide **20**. Addition of 3-chloro-propionyl chloride, in the presence of Et_3N, gave directly the Michael acceptor **12c** in 64% over two steps. Reaction of **12c** with morpholine, piperidine and ethyl piperidine-3-carboxylate furnished the adducts **13n**–**p** (Table 3).

The synthesis of the ketone-containing spiro-bicyclic imides **10**, **11d** and **12d** is illustrated in Figure 3. The enol ester[31] **7** was transformed initially into the corresponding α-nitro keto-ester by reaction with NH_4NO_3 in the presence of TFAA.[28] A subsequent chemoselective reduction of the nitro-group with Zn powder in EtOH and AcOH ensued, giving the amide **10** in 75% yield.[29] This keto-spiro lactam was reacted, on the one hand, with 2-bromoacetyl bromide to obtain imide **11d** and on the other hand with 3-chloro-propionyl chloride and Et_3N to provide the unsaturated imide **12d** (Fig. 3).

GABA transporter activity of the synthesized spirobicyclic derivatives was tested on HEK293 cell lines stably expressing GAT1, BGT1, GAT2 and GAT3 GABA transporter subtypes. Two of the tested inhibitors **12a** and **13d** showed selectivity towards GAT2 with low affinity (Table 4), alike all of the few GAT2 subtype-selective compounds found in a >500,000 entry-containing compound library.[7] Similar affinities of **12a** and **13d** indicate, that it is the acetyl-substituted azaspiro[4.5]decane moiety which endows these spirobicyclic compounds with GAT2 subtype-selectivity. This is important, because **13d** also contains a guvacine (**21**) analogue moiety. The acyl-substituted azaspiro[4.5]decane motif, however, has to adopt a definitive conformation to achieve GAT2-seletivity, since **13e**, a structure very similar to **13d** have lost subtype-specific inhibitory profile. Other compounds, **11a** and **13k** were found to be slightly selective towards BGT1 with moderate affinity (Table 4). The IC_{50} value of 164 μM displayed by **13k**, however, lags far behind the IC_{50} value of 0.59 μM of the most potent BGT1 inhibitor bicyclo[3.1.0]hexane derivative.[15] Compound **13e** displayed low affinity without selectivity, most probably due to the guvacine (**21**) ester moiety present in the structure.

On the BGT1 transporter **11a** has been found to be selective and modestly potent. Either removal of the oxo group from or addition of another oxo group to the spirobicyclic skeleton (**11c** or **11d**, respectively) hampered BGT1 activity. Substitution of Br with Cl (**11b**), I (**14**), C (**12a**), N_3 (**15**) or bulky heterocycles (**13a**–**j**) also abolished BGT1 inhibition. Apparently, the introduction of an ethylester group into the bulky heterocycle sidechain (**13k**), however, fully restored the BGT1 activity. The importance of the flexibility of the introduced O atoms and likely their distance to the N atom in the spirobicyclic skeleton is highlighted by the inactivity of **13p** in which a condensed heterocycle prevents the ester group from getting in close proximity to the spirobicyclic amine. Significantly, the comparison of the BGT1 selective and GAT2 selective structures, especially **13d** and **13k**, suggests that the spirobicyclic skeleton has the potential to confer GAT2 versus BGT1 transporter subtype selectivity. Furthering selectivity and efficacy allowed by the presence of spirobicyclic framework can lead to more effective investigational drugs.[32]

Due to the emerging roles, GAT2 plays in different diseases, novel chemical scaffolds aimed at identification of GAT2-selective structural motifs are of great (patho)-physiological and pharmacological relevance. Synthetic spirocyclicamine therapeutics with a range of size and charge-separation and biological activity are increasingly recognised. Based on GABA transport activity of azaspiro[4.5]decane carboxylates a new scaffold has been synthesized by developing three straightforward and efficient complementary routes. Biological evaluation of the novel scaffold synthesized this way discovered a subclass of a GAT2-selective compounds with acyl-substituted azaspiro[4.5]decane core.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.11.100.

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33. Spectral data for all compounds are provided in the Supplementary data including 1 Figure and Experimental Procedures and can be found with this article online.