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Original article

Enaminones 12. An explanation of anticonvulsant activity and toxicity per Linus Pauling's clathrate hypothesis

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ABSTRACT

The x-ray crystal structure of 3-((5-methylisoxazol-3-yl)amino)-5-methylcyclohex-2-enone (**12b**) and 3-((5-methylisoxazolyl-3-yl)amino)-5,5-dimethylcyclohex-2-enone (**12c**) were determined and correlated to their anticonvulsant activity in mice and rats. A hypothesis for the toxicity of the analogs are advanced. In addition, a series of 5-methyl-N-(3-oxocyclohex-1-enyl)-isoxazole-3-carboxamides were synthesized and evaluated for anticonvulsant activity. These compounds were compared to the activity of the corresponding amino and aminomethyl enaminones. Additional investigation involved the synthesis and evaluation of a trifluoromethyl analog of the active isoxazole tert-butyl 4-(5-methisoxazol-3-yl-amino)-6-methyl-2-oxo-cyclohex-3-ene carboxylate (**4f**).

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1. Introduction

Epilepsy is currently one of the most prevalent neurological disorders worldwide [1–3]. Despite the optimal use of available antiepileptic drugs, many patients with epilepsy fail to experience seizure control and others do so only at the expense of significant toxic side effects. Estimates suggest that available medications control the seizures in only 50% of patients or decrease the incidence in only 75% of patients [4]. The isoxazoles was our project of interest. Recently, gaboxadol (**1**) has shown to be a potent, selective extrasynaptic $GABA_A$ receptor agonist and has been investigated for its effect on a variety of clinical conditions (e.g. epilepsy, feeding disorders and pain [5–7]). In addition, Jansen et al. synthesized 5-(4-piperidyl)-3-isoxazolol (4-PIOL) (**2**), which proved to be a low-efficacy GABA mimetic, which was a weak partial agonist or antagonist, depending on the brain area and the $GABA_A$ receptor composition [8–10]. The isoxazole, N-[4,4-bis(3-methyl-2-thienyl)-3-butenyl]-3-hydroxy-4-methylamino-4,5,6,7-tetrahydrobenzo[d]isoxazol-3-ol (EF1 502, **3**) has been shown to selectively inhibit GAT1, GABA transporter 1, and GAT2/BGT-1, and also displayed anticonvulsant properties [11]. Recently our group [12] investigated the isoxazoles to see whether this group (**4, 5**) would have anticonvulsant properties in the enaminone series. We found that this series of analogs indeed provided anticonvulsant activity. Martinez and his group have generated a number of papers on the molecular modeling aspects of the data [13–15].

Abbreviations: MES, maximal electroshock seizure; TD_{50}, toxic dose for 50% of test animals; ED_{50}, effective dose for 50% of test animals; ip, intraperitoneal; PI, protective index, TD_{50}/ED_{50}; scPTZ, subcutaneous pentylenetetrazol; Tox, neurologic toxicity; ADD, Antiepileptic Drug Development; NINDS, National Institutes of Neurological Disorders and Stroke; ASP, Anticonvulsant Screening Program.

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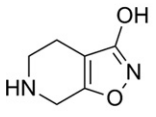

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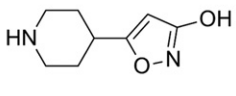

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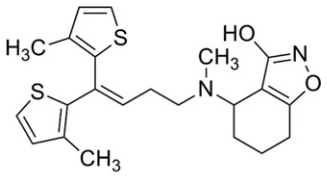

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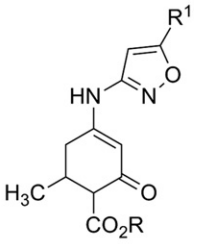
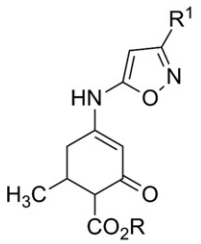


4

a. R=CH_3; R^1=H
b. R=C_2H_5; R^1=H
c. R=$C(CH_3)_3$; R^1=H
d.R=CH_3; R^1=CH_3
e. R=C_2H_5; R^1=CH_3
f. R=$C(CH_3)_3$; R^1=CH_3



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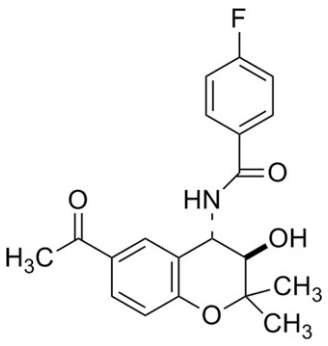
a. R=CH_3; R^1=CH_3
b. R=C_2H_5; R^1=CH_3
c. R=$C(CH_3)_3$; R^1=CH_3
d.R=CH_3; R^1=C_2H_5
e. R=C_2H_5; R^1=C_2H_5
f. R=$C(CH_3)_3$; R^1=C_2H_5

Carabesat (SB-204269, **6**), a novel fluorobenzoylamino benzopyran has been introduced which displays excellent



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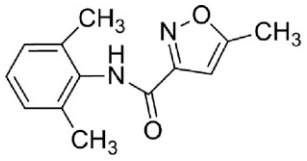

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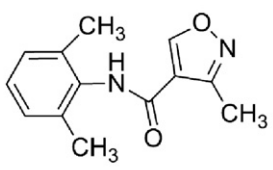

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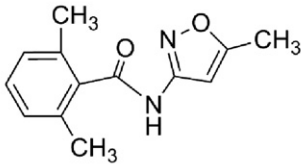

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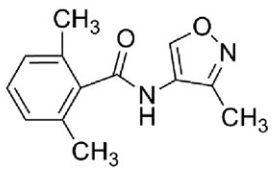

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anticonvulsant activity and efficacy comparable to existing anticonvulsants [3,16,17]. The benzamido pharmacophore as noted in structure **6** has been reported to be active against seizures [17]. Earlier, Lepage and coworkers [18,19] reported on a series of N-aryl isoxazolecarboxamides and their isomeric N-isoxazolylbenzamides (**7**–**10**) that were evaluated as potential anticonvulsants. In the carboxamide series **7** and **8,** were the most active in the maximal electroshock seizure (MES) animal test model, which is comparable to tonic-clonic seizures in humans. Compound **7** had an $ED_{50(rat)}$ of 5.5 mg kg^{-1}, a TD_{50} > 500 mg kg^{-1}, and a protective index (PI = ED_{50}/TD_{50}) > 90.9. Compound **8** provided an $ED_{50(rat)}$ of 8.9 mg kg^{-1}, a TD_{50} > 500 mg kg^{-1} and a PI > 56.2. In the isomeric benzamide series **9** and **10**, however, the results were less clear. Compound **9**, 2,6-dimethyl-N-(5-methylisoxazol-3-yl)benzamide, had an $ED_{50(rat)}$ of 9.2 mg kg^{-1}, a TD_{50} > 500 mg kg^{-1}, and a PI > 54.3. Compound **10** was found inactive. Compound **9** (D2916)

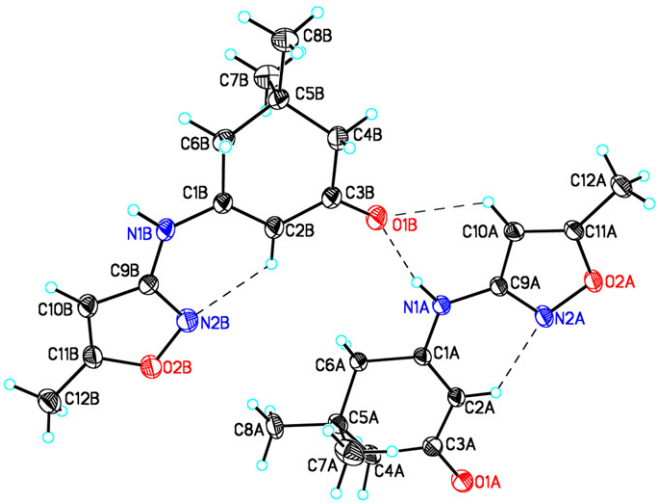


Fig. 1. X-ray crystal structure of 3-((5-methylisoxazol-3-yl)amino)-5,5-dimethylcyclohex-2-enone (**12c**).

was advanced for further evaluation and displayed different pharmacokinetic profiles in male and female rats while compound **7** (D2624) was advanced to preclinical testing [19,20].

In our continuing investigations on the enaminones, we evaluated a series of comparable 3- and 5-methyl isoxazoles [12]. Our results paralleled those of Lepage and coworkers in that the 5-methyl isoxazole compounds, **4**, were highly active. Compound **4e**, $R = C_2H_5$ had an $ED_{50(rat)}$ of 68.9 mg kg^{-1}, a $TD_{50} > 500$ mg, and a PI > 7.3. For compound **4f**, $R = C(CH_3)_3$, the $ED_{50(rat)}$ was 28.1 mg kg^{-1}, the $TD_{50} > 500$ mg kg^{-1}, and the PI > 17.8. For the isomeric 3-methyl series, **5a**, $R = CH_3$ and **5c**, $R = C(CH_3)_3$ were orally active at 30 mg kg^{-1}. However as a group, the 3-methyl isoxazoles were the more toxic entities. It was of interest to determine whether the amino enaminone pharmacophore could be extended to the amide series as well. Earlier, Foster et al. investigated the anticonvulsant activity of vinylic benzamides and their comparable benzylamines [21]. The evaluation revealed significant differences in anticonvulsant activity between the two entities. In addition to noting the pharmacological changes in converting the enaminones into amide derivatives, Foster was interested in evaluating substituents at position 6 of the cyclic enaminone system. We found the 6-methyl substituent to have augmented activity. In continuing with our extensive SAR study, we replaced the 6-methyl group with a 6-trifluoromethyl group. While both groups are lipophilic and spatially, they are relatively the same size, but electronically, these groups are quite different, with the trifluoromethyl group being electronegative and the methyl group being electropositive.

Linus Pauling, in order to explain the diversity of structures that provided general anesthesia, put forth the clathrate (L. clathare, to furnish with a lattice) hypothesis. Pauling hypothesized that all of the compounds that possessed general anesthetic properties were capable of producing clathrates in the brain, which interfered with normal brain transmission [22–24]. We noted that a clathrate had formed (Fig. 1) in 3-((5-methylisoxazolyl-3-yl) amino)-5,5-dimethylcyclohex-2-enone **12c**. This structure may be the cause of the toxicity noted in the animal testing of the compound.

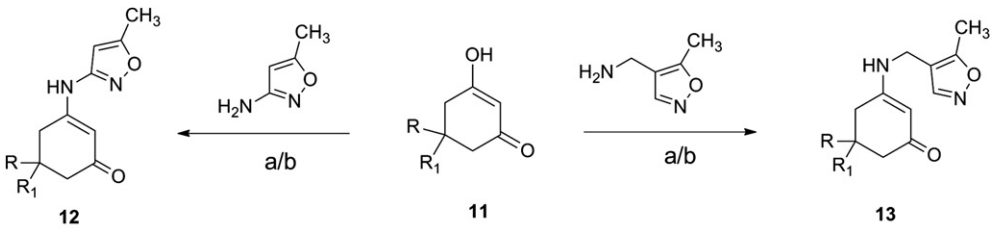
2. Results and discussion

2.1. Chemistry

The 5-methyl isoxazole enaminone analogs were synthesized as shown in Scheme 1 and have been previously documented in our laboratories [12] and elsewhere [26]. Ketones **11a** ($R = R^1 = H$), **11b** ($R = CH_3$, $R^1 = H$), **11c** ($R = R^1 = CH_3$), were either commercially available (**11a** and **11c**) or synthesized (**11b**) via an ester hydrolysis and decarboxylation reaction of the *tert*-butyl ester diketone [25,26].

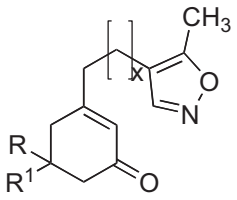
The properties of products **12a–c** and **13a,b** are provided in Table 1. Scheme 2 shows the process by which we produced the 3-carboxamide isoxazole analogs. We recently reported on the base-catalyzed solution phase synthesis of vinylic benzamides [27] that was employed in the current synthesis. Employing sodium hydride (NaH), N-acylation provided a smoother condensation reaction without the need for extensive workup and column chromatography as was previously reported using triethylamine [21]. The properties of products **15a–c** are provided in Table 2. The ketones in Scheme 1 were used in this synthesis. They were aminated by means of ammonium acetate (NH_4OAc) in refluxing benzene or toluene using a Dean–Stark trap, providing 3-amino vinylogous amide analogs **14a–c**. The use of ammonium acetate was more efficient and less hazardous from our previous method that utilized gaseous ammonia [21]. After purification, the amines were subsequently condensed with 5-methyl-isoxazole-3-carbonyl chloride, prepared as reported by Lepage and coworkers [18], forming the desired 5-methly-N-(3-oxocyclohex-1-enyl)-isoxazole-3-carboxamides **15a–c**.

The synthesis of the trifluoromethyl analog **19** was a variation of the Friary procedure [26] utilizing ethyl 4,4,4-trifluorocrotonate **16** which was condensed with tert-butyl acetoacetate **17** in the presence



Scheme 1. Synthesis of the 5-methyl-isoxazole enaminones. Conditions: a/b EtOH:EtOAc (1:1), Δ 6 h.

Table 1
5-methyl isoxazole enaminones **12a**–**c**, **13a**,**b**.



Compound	*R*	R^1	x	% Yield	mp, °C	Clog P[a]
12a	H	H	0	80	225–226	−0.03
12b	CH_3	H	0	51	205–206	0.49
12c	CH_3	CH_3	0	51	204–207	1.01
13a	CH_3	H	1	73	119–120	0.30
13b	CH_3	CH_3	1	62	167–168	0.82

[a] Calculated from reference [36].

of sodium ethoxide (NaOEt), producing tert-butyl 6-(trifluoromethyl)-4-hydroxy-2-oxocyclohex-3-ene carboxylate **18**, which was subsequently condensed with 3-amino-5-methylisoxazole under previously reported conditions [25] to yield tert-butyl 4-(5-methylisoxazole-3-ylamino)-6-(trifluoromethyl)-2-oxocyclohex-3-ene carboxylate **19**. The synthesis is shown in Scheme 3.

High field NMR analyses on the reported compounds were consistent with the assigned structures.

2.2. Pharmacology

Pharmacological testing of the compounds listed in Tables 1 and 2 have been provided by the Antiepileptic Drug Development (ADD) Program, Epilepsy Branch, Neurological Disorders Program, National Institute of Neurological Disorders and Stroke (NINDS) [28]. Phase I evaluation of the reported compounds involved three tests: maximal electroshock seizure (MES), subcutaneous pentylenetetrazol (scPTZ), and neurologic toxicity (Tox) in mice. Phase I data for the compounds are shown in Table 3. As previously reported [28], to differentiate the results between different rodent species, the most active class 1 analogs in Phase I were subsequently evaluated for oral (po) activity (Phase VIA, Phase VIB) in the rat. All data is shown in Table 3. Phase VIB determined the median dose (ED_{50}) and median toxic dose (TD_{50}) in male Sprague–Dawley rats. The ED_{50} and TD_{50} values and their confidence limits were determined at the time of peak effect for **12b** by the method of Litchfield and Wilcoxon [29].

2.3. X-ray crystallography

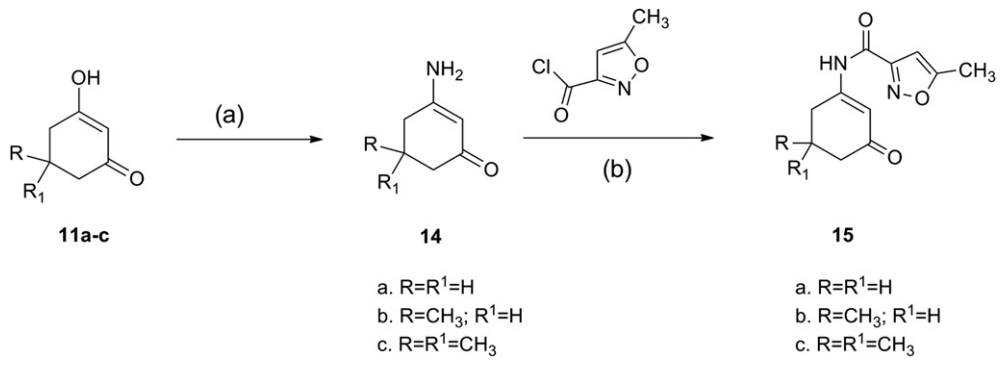
As a verification of our previously document role of x-ray analysis in the evaluation of anticonvulsant activity [30–35], an x-ray diffraction study of the representative 5-methyisoxazoles was undertaken. This data is shown in Figs. 1 and 2 for **12c** and **12b**, and Tables 4 and 5, respectively.

2.4. Structure–activity correlations

2.4.1. Enaminones (*12a–c, 13a,b*)

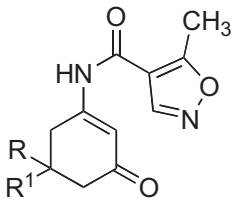
Enaminones **12a**–**c** provides dramatic variations in activity and toxicity. In the Phase I evaluation in mice, **12a** was inactive in the MES evaluation while showing exclusive activity in the scPTZ test, a departure from our previous reported profiles of the enaminone derivatives. This compound, **12a**, when evaluated in the rat provided broad protection in both test models, i.e. MES and scPTZ. It was also noted that the protection was greater by the intraperitoneal (ip) route than orally, indicating a possible absorption problem. CLog P data [36] (Table 1) indicates that **12a** is the most hydrophilic of the analogs evaluated. The addition of a methyl group to the cyclohexene ring, as in **12b**, produces a compound with dual protection in mice, however, with increased neurological toxicity in mice. Compound **12b** was evaluated in the rat (ip) and provided an MES ED_{50} 66 mg kg^{-1}, a TD_{50} >120 mg kg^{-1} and a PI 1.8. Solubility was a problem with this compound as well. In the ip evaluation in rats at the highest dose, the volume of the injected solution affected the apparent activity of the compound (2/8 protected at the smaller volume vs. 6/8 protected with a doubled volume). The dimethyl substituted analog, **12c**, produced a compound that was exclusively MES active and toxic. It should be noted that the starting amines **14a**–**c** (Table 3) were all quite toxic as well. Analysis of the x-ray structures of **12b** and **12c** display significant differences. In Fig. 2, **12b** shows the characteristic intramolecular hydrogen binding of the vinyl proton at C2 with the isoxazole ring nitrogen, N2, producing a pseudo three-ring configuration as previously reported [21].

However, in Fig. 1, a more complicated structural configuration occurs. The dimethyl analog, **12c**, is assembled as a head-to-tail dimer, exhibiting both intramolecular hydrogen bonding (C2B-N2B and N2A-C2A), but also intermolecular hydrogen bonding with the carbonyl oxygen (O1B and the isoxazoles proton, C10A, and the proton on the secondary amine, N1A) producing a pocket between these molecules. Further, this clathrate formation effectively blocks



Scheme 2. Synthesis of the 3-carboxamide 5-methyl-isoxazole analogues. Conditions: (a) NH_4OAc, Δ 1 h. (b) NaH, dry THF, 20 °C.

Table 2
3-carboxamide isoxazole enaminones **15a–c**.



Compound	*R*	R^1	% Yield	mp, °C	CLog P[a]
15a	H	H	23	132–134	−0.22
15b	CH_3	H	21	175–177	0.30
15c	CH_3	CH_3	35	144–146	0.82

[a] Calculated from reference [36].

access to the proposed active site [37–39] by virtue of the dimethyl substituents at both ends of the pocket, although hydrogen bonding has been correlated with anticonvulsant activity. Poupert and coworkers observed that the anticonvulsant activity of phenytoin [40] was greatly reduced when the H-bonding groups (C=O or NH) were removed from this compound. In contrast to the previous active analogs, the increase in chain length abolished activity. It is worth noting that in the homologous benzene series, i.e. in going from aniline to benzylamine [21,25a] activity increased. However, in contrast with the previous series, no toxicity was observed.

Molecular modeling studies were undertaken to determine the probability of the pseudo ring system forming in this series as compared with the previously reported isoxazole analogs. Employing our previous documented analysis [38] the following vinyl proton to the isoxazole nitrogen bond distances were determined: **12b** 2.50 Å vs. **13a** 3.66 Å (Δ 1.16 Å) and **12c** 2.38 Å vs. **13b** 4.49 Å (Δ 2.11 Å). These significant differences may account for the lack of activity in this series. Clog P determinations (Table 1) show an increase in lipophilicity in the **13a,b** series which would be favorable for anticonvulsant activity but the effective interacting bond distance is apparently the limiting factor.

Clog P determines both transportability through biological membranes and pharmacological effects by binding to the receptor [13]. The optimum Clog P should be less inhibited in their movement through the aqueous and lipophilic phases of living tissue. We note the Clog P for our compounds is in the optimum range [41].

2.4.2. Amides (*15a–c*)

Cyclohexyl amide **15a** showed some protective activity in the scPTZ model, but it occurred at a dose that produced toxic side effects. The 5-methyl derivative, **15b**, was also inactive and produced more toxic effects (Table 3). The 5,5-dimethyl analog, **15c** was also inactive, but without any neurotoxicity. Clearly the amides did not offer any notable anticonvulsant leads.

2.4.3. Trifluoromethyl analog (*19*)

The trifluoromethyl analog, **19**, resulted in an inactive and non-toxic compound. Therefore, replacement of the methyl group with an electronegative substituent at the position 6 with the same approximate size abolishes activity. To resolve the actual lipophilicity of the 6-trifluoromethyl intermediate **18**, (Clog P 1.41 vs. 2.00 for the 6-methyl analog), we experimentally determined these analogs. The actual log P was 0.28 for **19** vs. −0.16 for the 6-methyl analog, **4f**. Earlier we had shown [25a] that replacement of the methyl group with a bulky substituent, e.g. phenyl, abolishes activity, while replacement with hydrogen maintains activity. It is therefore evident that substitution on the 6 position is critical for anticonvulsant activity.

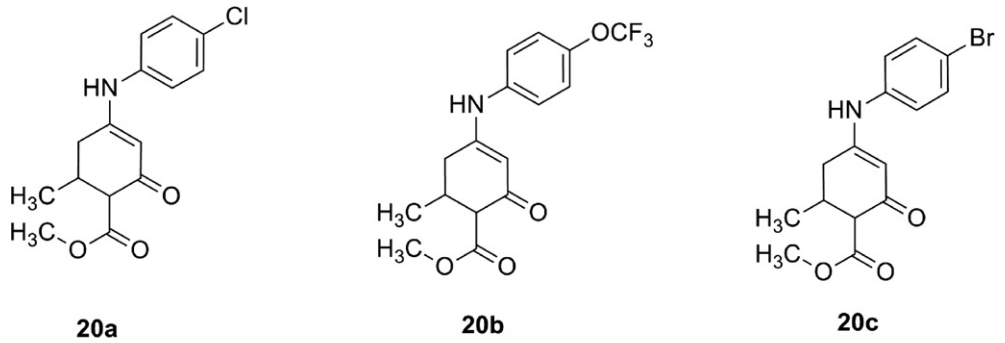
2.5. Mechanism of action

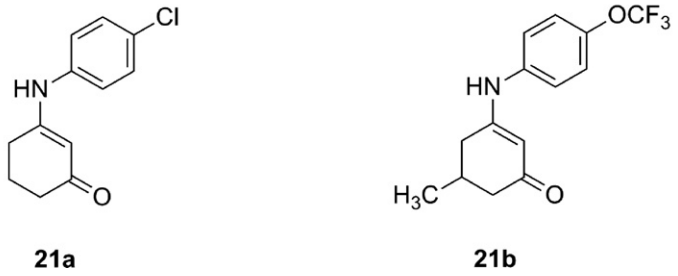
We had earlier shown that for the enaminones methyl 4-[(4′-chlorophenyl)-amino]-6-methyl-2-oxocyclohex-3-ene-1-carboxylate **20a**, and methyl 4[(4′-trifluoromethoxyphenyl)amino]-6-methyl-2-oxocyclohex-3-ene-1-carboxylate **20b**, were active as inhibitors of voltage-gated sodium channels [25c]. New information has recently

Table 3
Anticonvulsant screening project (ASP): Anticonvulsant results.

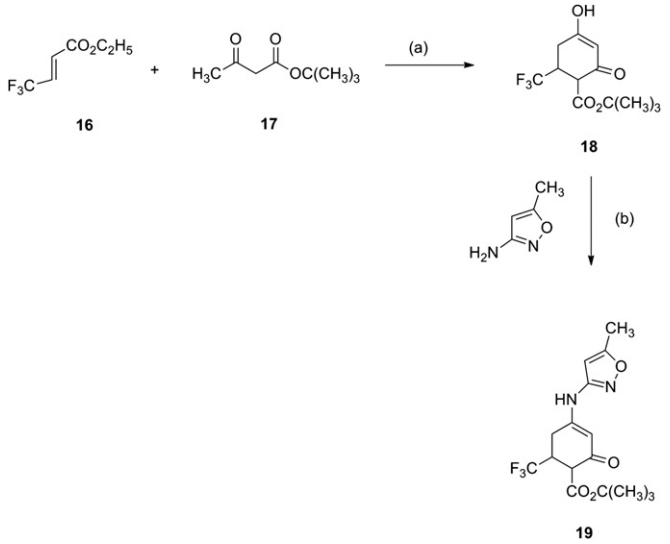
Compound	Anticonvulsant results[a]
12a	Phase I (mice): Class 1 MES test-inactive up to 300 mg kg^{-1}; scPTZ test-active 4/5 animals protected at 300 mg kg^{-1} at 30 min; Tox test-no toxicity up to 300 mg kg^{-1} at 30 min and 4 h. Phase VIA (i.p. rats): MES test-active 2/3 animals protected at 30 mg kg^{-1} at 1 h; scPTZ test-active 1/3 animals protected at 30 mg kg^{-1} at 4 h; Tox test-0/6 animals displayed toxicity at 30 mg kg^{-1} up to 4 h.
12b	Phase I (mice): Class 1 MES test-active 1/3 animals protected at 100 mg kg^{-1} at 15 min, 2/3 at 300 mg kg^{-1} at 1 h; scPTZ test-active 4/5 animals protected at 300 mg kg^{-1} at 30 min; Tox test-active 3/8 animals were unable to grasp rotorod at 100 mg kg-1 at 30 min and 3/4 unable o grasp rotorod at 300 mg kg-1 at 15 min. Phase VIB (i.p. rats): MES ED_{50} 66 mg kg^{-1}, TD_{50} > 120 mg kg^{-1}; PI 1.8.
12c	Phase I (mice): Class 1 MES test-active 3/7 animals protected at 100 mg kg^{-1} at 30 min, 4/5 animals protected to 300 mg kg^{-1} at 30 min and at 4 h; scPTZ test-inactive up to 300 mg kg^{-1}; Tox test-2/8 animals were toxic at 100 mg kg^{-1} at 30 min, 3/4 toxic at 300 mg kg^{-1} at 30 min and 1/2 toxic at 300 mg kg^{-1} at 4 h.
13a	Phase I (mice): Class 3
13b	Phase I (mice): Class 3
14a	Phase I (mice): Class 3 (in Tox evaluation-8/8 animals displayed muscle spasms, and unable to grasp rotorod at 100 mg kg^{-1}; 4/4 animals showed the same toxicity at 300 mg kg^{-1} at 30 min)
14b	Phase I (mice): Class 3 (in Tox evalution-8/8 animals displayed muscle spasms at 100 mg kg^{-1}; 4/4 animals displayed muscle spasms and unable to grasp rotorod at 300 mg kg^{-1} at 30 min)
14c	Phase I (mice): Class 3 (in scPTZ evaluation-1/1 animals died following continuous seizure at 30 mg kg^{-1} at 4 h; 1/1 animals died following continuous seizure at 30 mg kg^{-1} at 30 min; 1/5 animals underwent myoclonic jerks at 100 mg kg^{-1} at 4 h; (in Tox evaluation-4/8 animals displayed muscle spasms at 100 and 300 mg kg^{-1} at 30 min)
15a	Phase I (mice): Class 3
15b	Phase I (mice): Class 3; 4/4 mice unable to grasp the rotorod at 300 mg kg^{-1} at 30 min; 2/2 mice toxic at 300 mg kg^{-1} at 4 h
15c	Phase I (mice): Class 3
19	Phase I (mice): Class 3

[a] Phase I in mice activity-class 1 = activity at 100 mg kg^{-1} or <; class 2 = activity between 100 and 300 mg kg^{-1} class 3 = no activity at 300 mg kg^{-1}.





been reported by Kombian et al. [39] that two of our analogs, methyl 4-(4′-bomophenylamino)-6-methyl-2-oxocyclohex-3-ene carboxylate (ADD 206038) **20c** [25b], and 3-(4′-chlorophenylamino)cyclohex-3-enone **21a**, support an indirect action on the GABA system, producing GABA-dependent synaptic depression. Thus, these enaminones may act to potentiate extracellular GABA concentration that will promote GABA acting on $GABA_A$ receptors, located on presynaptic glutamate terminals to decrease EPSC amplitude [39]. We have recently evaluated similar analogs that we used in the sodium channel inhibition studies plus an additional compound 5-methyl-3-((4-(trifluoromethoxy)phenyl)amino)cyclohex-2-enone **21b** for GABA activity using mouse olfactory bulb slices and found that the latter



Scheme 3. Synthesis of the 3-amino-5-methyl-isoxazole trifluoromethyl analog. Conditions: (a) Na, dry EtOH, Δ 2.5h; (b) EtOAc: EtOH (1:1), Δ 6 h.

Table 4
Atomic coordinates ($\times 10^4$) and equivalent isotropic displacement parameters ($A^2 \times 10^3$) for 3-((5-methyisoxazol-3-yl)amino)-5,5-dimethylcyclohex-2-enone (**12c**). U(eq) is defined as one third of the trace of the orthogonalized U_{ij} tensor.

	x	y	z	U(eq)
O(1A)	9359(4)	−3545(2)	8474(2)	78(1)
O(2A)	715(4)	−4235(2)	6360(2)	64(1)
O(1B)	543(5)	−8416(2)	6673(2)	90(1)
O(2B)	6300(4)	−10,220(2)	9011(2)	75(1)
N(1A)	3351(5)	−6267(2)	7016(2)	54(1)
N(2A)	2523(5)	−4516(2)	6781(2)	65(1)
N(1B)	1228(5)	−11,531(2)	7928(2)	60(1)
N(2B)	4459(5)	−10,190(2)	8476(2)	73(1)
C(1A)	5231(5)	−5908(2)	7529(2)	45(1)
C(2A)	6291(5)	−4821(3)	7765(2)	51(1)
C(3A)	8308(6)	−4532(3)	829(2)	55(1)
C(4A)	9177(5)	−5451(3)	8608(2)	58(1)
C(5A)	7461(6)	−6497(3)	8639(2)	57(1)
C(6A)	6109(6)	−6863(3)	7795(2)	55(1)
C(7A)	5984(7)	−6207(4)	9320(2)	92(2)
C(8A)	8595(7)	−7448(3)	8808(3)	97(2)
C(9A)	2015(5)	−5618(3)	6684(2)	46(1)
C(10A)	−12(5)	−6090(3)	6214(2)	51(1)
C(11A)	−754(5)	−5201(3)	6034(2)	50(1)
C(12A)	−2771(6)	−5065(3)	5585(2)	63(1)
C(1B)	213(5)	−10,962(3)	7437(2)	51(1)
C(2B)	1017(6)	−9907(3)	7321(2)	59(1)
C(3B)	−183(6)	−9366(3)	6812(2)	62(1)
C(4B)	−2466(6)	−9960(3)	6453(2)	66(1)
C(5B)	−2745(6)	−11,243(3)	6248(2)	55(1)
C(6B)	−1968(5)	−11620(3)	7024(2)	57(1)
C(7B)	−1445(7)	−11,622(3)	5526(3)	82(1)
C(8B)	−5160(6)	−11,776(3)	5998(3)	81(1)
C(9B)	3225(6)	−11,191(3)	8408(2)	54(1)
C(10B)	4144(6)	−11,880(3)	8867(2)	62(1)
C(11B)	6029(6)	−11,243(3)	9230(2)	62(1)
C(12B)	7822(6)	−11,417(3)	9793(2)	78(1)

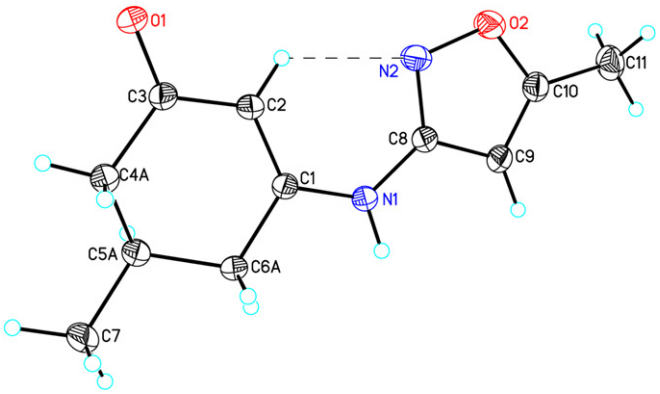


Fig. 2. X-ray crystal structure of 3-((5-methylisoxazol-3-yl)amino)-5-methylcyclohex-2-enone (**12b**).

compound acts as a positive allosteric modulator of $GABA_A$ receptors and enhances GABA affinity [42].

3. Experimental section

3.1. Chemistry

Melting points were determined on a Thomas-Hoover capillary melting point apparatus and were uncorrected. Observed boiling points were also uncorrected. Analytical TLC was performed using silica gel with a fluorescent indicator coated on 1 × 3 inch glass plates with 0.2 mm thickness (Whatman MKGF silica gel 200 μ). IR spectra were obtained on a Nicolet Magna-IR 560 spectrometer. The samples were recorded with KBr pellets. The ^1H and ^{13}C NMR spectra were determined on a Bruker 1 Ultra Shield-400 MHz NMR spectrometer. The samples were dissolved either in deuterated chloroform ($CDCl_3$) or dimethylsulfoxide ($DMSO\text{-}_{d_6}$) containing 0.03% tetrametylsilane (TMS) as an internal reference. Elemental analyses (C, H, and N) were determined by Schwarzkopf Microanalytical Laboratory, Woodside, NY 11377, USA. The analytical results for the elements were within ±0.4% of the theoretical values. Cyclohexane-1,3-dione, 5,5-dimethylcyclohexane-1,3-dione and 3-amino-5-methyisoxazole were obtained from Aldrich Chemical Company and used without further purification. (5-Methylisoxazole-3-yl)methaneamine was obtained from ASDI Biosciences, Inc. (http://www.asdi.net), or prepared synthetically [43]. 5-Methylcyclohexane-1,3-dione, [12,26] 3-aminocyclohex-2-enone (**14a**, $R = R = H$), [17,27] 3-amino-5-methylcyclohex-2-enone (**14b**, $R^1 = CH_3$, $R = H$) [18,27] and 3-amino-5,5-dimethylcyclohex-2-enone (**14c**, $R^1 = R = CH_3$) [44] were prepared by literature methods.

Table 5
Atomic coordinates ($\times 10^4$) and equivalent isotropic displacement parameters ($A^2 \times 10^3$) for 3-((5-methyisoxazol-3-yl)amino)-5-methylcyclohex-2-enone (**12b**). U(eq) is defined as one third of the trace of the orthogonalized U_{ij} tensor.

	x	y	z	U(eq)
O(1)	−1274(1)	−143(2)	3070(2)	64(1)
O(2)	−1456(2)	5913(2)	952(2)	72(1)
N(1)	1307(2)	3667(2)	2452(2)	48(1)
N(2)	−887(2)	4572(2)	1549(2)	68(1)
C(1)	1119(2)	2289(2)	2824(2)	43(1)
C(2)	−73(2)	1709(2)	2619(2)	47(1)
C(3)	−195(2)	311(2)	3133(2)	48(1)
C(4A)	1015(3)	−6164(4)	3783(2)	52(1)
C(5A)	2124(2)	−237(2)	3403(2)	51(1)
C(6A)	2358(2)	1403(2)	3489(2)	48(1)
C(4B)	1010(60)	−600(80)	3470(60)	52(1)
C(5B)	2300(30)	160(40)	4210(40)	51(1)
C(6B)	2180(30)	1420(40)	4980(30)	48(1)
C(7)	3382(2)	−1093(3)	4143(3)	71(1)
C(8)	373(2)	4736(2)	1847(2)	45(1)
C(9)	668(2)	6152(2)	1469(2)	52(1)
C(10)	−499(2)	6798(2)	918(2)	53(1)
C(11)	−931(3)	8253(3)	310(2)	71(1)

3.2. General procedure for isoxazole enaminones

3.2.1. 3-((5-Methylisoxazol-3-yl)amino)cyclohex-2-enone (*12a*)

Cyclohexane-1,3-dione (**11a**, $R^1 = R = H$, 3.03 g, 27 mmol) and 3-amino-5-methylisoxazole (3.24 g, 33 mmol) were added to a mixture of absolute EtOH (100 mL) and EtOAc (100 mL), and the solution was refluxed and stirred for 6 h. During that time, one-half of the solvents were slowly removed, via a Dean–Stark trap, and after cooling, replaced with an equal volume of anhydrous Et_2O. The mixture became cloudy while stirring and was allowed to continue overnight whereupon crystals spontaneously deposited. Recrystallization from EtOAc yielded a tan powder, 4.15 g (80%); mp 225–226 °C; ^1H NMR ($DMSO\text{-}_{d6}$) δ 1.1–2.2 (6H, m, cyclohexene ring), 3.4 (3H, s, CH_3), 6.0 (1H, s, =CH), 6.3 (1H, s, isoxazole H), 9.5 (1H, br s, NH). ^{13}C ($DMSO\text{-}_{d_6}$) δ 12.30, 17.75, 21.96, 38.43, 96.22, 106.22, 158.06, 160.18, 168.95. 197.81. IR (KBr) 3339.41 cm^{-1} (NH stretch), 3140.90 cm^{-1} (5-methylisoxazole stretch), and 1685.35 cm^{-1} (C=O stretch). Anal. (C, H, N).

3.2.2. 3-((5-Methylisoxazole-3-yl)amino)-5-methylcyclohex-2-enone (*12b*)

Following the general procedure for **12a**, ketone (**11b**, $R^1 = CH_3$, $R = H$) and 3-amino-5-methylisoxazole produced **12b** as pale yellow crystals from EtOAc, 2.81 g (50.5%); mp 205–206 °C; ^1H NMR ($DMSO\text{-}_{d6}$) δ 1.0 (3H, d = 6.4 Hz, CH_3), 1.9–2.4 (5H, m, cyclohexene ring), 3.3 (3H, s, CH_3 on isoxazole ring), 6.0 (1H, s, =CH), 6.3 (1H, s, isoxazole H), 9.4 (1H, br s, NH). ^{13}C ($DMSO\text{-}_{d_6}$) δ 11.21, 32.08, 42.03, 46.04, 49.98, 53.36, 95.46, 102.90, 105.15, 168.81, 197.40. IR (KBr) 3342.75 cm^{-1} (NH stretch), 3139.76 cm^{-1} (5-methylisoxazole stretch), and 1680.15 cm^{-1} (C=O stretch). Anal. (C, H, N).

3.2.3. 3-((5-methylisoxazol-3-yl)amino)-5,5-dimethylcyclohex-2-enone (*12c*)

Following the general procedure for **12a**, ketone (**11c**, $R^1 = R = CH_3$) and 3-amino-5-methylisoxazole produced **12c** as light yellow crystals from EtOAc, 3.06 g (51.4%); mp 204–207 °C; ^1H NMR ($DMSO\text{-}_{d6}$) δ 1.0 (6H, s, gem CH_3), 2.0 (2H, s, C_4 CH_2), 2.5 (2H, s, C6 CH_2), 3.3 (3H, s, CH_3 on isoxazole ring), 6.0 (1H, s, =CH), 6.2 (1H, s, isoxazole H), 9.4 (1H, br s, NH). ^{13}C ($DMSO\text{-}_{d_6}$) δ 5.02, 27.34, 41.94, 43.43, 45.47.47.23, 97.09, 102.34, 105.25, 155.33, and 197.05. IR (KBr) 3340.45 cm^{-1} (NH stretch), 3143.68 cm^{-1} (5-methylisoxazole stretch), and 1678.55 cm^{-1} (C=O stretch). Anal. (C, H, N).

3.2.4. 3-[(5-Methylisoxazol-4-yl)methylamino]-5-methylcyclohex-2-enone (*13a*)

Into a 50 mL single neck flask, 5-methyl-1,3-cyclohexanedione (0.88 g, 7 mmol) and 5-methyl-3-isoxazolemethylamine (0.95 g 8.5 mmol), were added to a mixture of absolute EtOH (25 mL) and EtOAc (25 mL), and the solution was refluxed and stirred for 6 h. During refluxing, one-half of the solvent mixture was slowly removed, via a Dean–Stark trap. Once cooled to r.t. an equal volume of anhydrous Et_2O was added. After the addition of ether, the mixture became cloudy and was allowed to stir overnight at room temperature whereupon crystals spontaneously deposited. The mixture was filtered and dried to give the title compound, **13a**, as

yellow crystals, mp 119–120 °C, unchanged after recrystallization from EtOAc (1.12 g, 73%); ^{1}H NMR (DMSO-$_{d_6}$) δ 1.1 (3H, d, CH_3), 1.8 (2H, s, C_4 CH_2), 2.1–2.4 (5H, m, cyclohexene ring), 2.5 (3H, s, isoxazole CH_3), 4.4 (1H, s, cyclohexene CH=), 5.2 (1H, s, isoxazole CH=), 6.0 (1H, s, NH). ^{13}C (DMSO-$_{d6}$) 12.45, 20.35, 29.29, 36.44, 44.27, 96.07, 100.01, 159.89, 165.38, 170.44 and 197.29. IR (KBr) 3347.25 cm^{-1} (NH stretch), 3183.44 cm^{-1} (5-methylisoxazole stretch) and 1688.45 cm^{-1} (C=O stretch). Anal. (C, H, N).

3.2.5. 3-[(5-Methylisoxazol-4-yl)methylamino]-5,5-dimethylcyclohex-2-enone (**13b**)

In a similar manner, the amination reaction proceeds as previously stated in **13a**. After the addition of ether, the mixture became cloudy and was allowed to stir overnight at room temperature whereupon crystals spontaneously deposited. The mixture was filtered and dried to give the title compound, **13b**, as tan crystals mp 167–168 °C, unchanged after recrystallization from EtOAc (0.49 g, 62%); ^{1}H NMR (DMSO-$_{d6}$) δ 1.0 (6H, s, gem CH_3), 1.1 (2H, 2, C_4 CH_2), 2.2–2.3 (5H, m, cyclohexene ring), 2.4 (3H, s, isoxazole CH_3), 4.3 (1H, s, cyclohexene CH=), 5.2 (1H, s, isoxazole CH=), 6.0 (1H, s, NH). ^{13}C (DMSO-$_{d_6}$) 11.18, 28.29, 32.33, 38.47, 42.20, 50.44, 95.32, 100.44, 159.89, 163.58, 170.22 and 197.32. IR (KBr) 3447.25 cm^{-1} (NH stretch), 3203.44 cm^{-1} (5-methylisoxazole stretch), and 1708.45 cm^{-1} (C=O stretch). Anal. (C, H, N).

3.2.6. 3-Aminocyclohex-2-enone (**14a**)

Into a 500 mL round bottom flask fitted with a Dean–Stark trap and a magnetic stirrer was added 150 mL of anhydrous benzene, 1,3-cyclohexanedione (5.6 g, 0.05 mol), and ammonium acetate (7.7 g, 0.1 mol). The mixture was stirred continuously, and refluxed for 5 h. The reaction was allowed to reach room temperature. The precipitate was recrystallized with ethyl acetate to afford yellow crystals (1.73 g, 31% yield). Mp-128–131 °C [lit. 128–131 °C (35)]. ^{1}H NMR Acetonitrile-d_3) δ −1.8–2.4 (6H, m, cyclohexene ring), 5.1 (1H, s, CH=). 5.3–5.5 (2H, s, br, NH_2).

3.2.7. 3-Amino-5-methylcyclohex-2-enone (**14b**)

In a like manner as previously stated in **14a**, the title compound, **14b**, was produced in 80% yield, yellow powder, mp, 175–177 °C, ^{1}H (DMSO-d_6) δ 0.8–1.2 (3H, d, J = 2.4 Hz, CH3), 1.7–2.6 (5H, m, cyclohexene ring), 4.9 (1H, s, CH=) 6.5–7.0 (2H, s, br, NH_2).

3.2.8. 3-Amino-5,5-dimethylcyclohex-2-enone (**14c**)

In a like manner as previously stated in **14a**, the title compound, **14c**, was produced in 36% yield as light yellow crystals, mp 156–158 °C, ^{1}H NMR (acetone-d_6) 1.0 (6H, s, gem CH_3), 2.2 (2H, s, C_6 CH_2), 2.9 (2H, s, C_4 CH_2) 6.0–6.2 (2H, s, br, NH_2).

3.2.9. 5-Methylisoxazole-3-carboxylic acid

Sodium bicarbonate (13.2 g, 0.157 mol), hydroxylamine hydrochloride (10.91 g, 0.157 mol) and ethyl 2,4-dioxopentanoate (25 g, 01.57 mol) were added to 107 mL of ethanol in a 500 mL round bottom flask. The mixture was refluxed for 4 h. The precipitate was filtered and the filtrate concentrated in vacuo to yield the ester. The ester was dissolved in 53.5 mL of EtOH to which was added sodium hydroxide (59 mL, 10% solution). The solution stirred overnight at r.t.. The solvent was evaporated under reduced pressure. The sodium salt was dissolved in water and acidified with concentrated hydrochloric acid to precipitate the title compound. Recrystallization of the crude product from EtOAc yielded the product, white crystals, mp 172–174 °C (lit. 182 °C (7), 79%, ^{1}H NMR (DMSO-d_6) 2.3 (3H, s, CH_3), 6.6 (1H, s, CH=), 7.0 (1H, s, COOH). IR spectrum (KBr) 5-methylisoxazole stretch at 3149 cm^{-1} and C=O stretch at 1655.35 cm^{-1}. Anal: (C, H, N, O).

3.2.10. 5-Methylisoxazole-3-carbonyl chloride

A mixture of 5-methylisoxazole-3-carboxylic acid (3.81 g, 0.03 mol) in 150 mL of toluene was dried via azeotropic distillation of some of the toluene into a two-neck 500 mL round bottom flask fitted with a Dean–Stark trap and a magnetic stirrer. The mixture was allowed to cool to about 90 °C, and dimethylformamide, 1 mL, was added. Thionyl chloride (4.8 g, 0.04 mol) was added dropwise over 15 min and the mixture was refluxed for 5 h with continuous stirring. The reaction was allowed to cool to room temperature, and then the solvent was evaporated in vacuo to yield the product, a dark-brown oil, 80%, which crystallized on standing. It was kept under vacuum until further use.

3.3. General procedure for isoxazole-3-carboxamides

3.3.1. 5-Methyl-N-5-methyl-3-oxocyclohex-1enyl)isoxazole-3-carboxamide (**15b**)

To a 250 mL single neck flask equipped with a ST "Y" tube, condenser and a pressure equalizing dropping funnel containing 85 mL of anhydrous THF, was cautiously added NaH (0.84 g, 35.0 mmol) with constant stirring, maintaining the temperature below 20 °C with external cooling. After the reaction, **14b** (1.9 g, 15.0 mmol) was added over 30 min through the "Y" tube, and after the addition, a further 10 mL of THF washed the "Y" tube. The reaction mixture was heated to reflux for 20 min, cooled to room temperature and 5-methylisoxazolyl carbonylchloride [7] (2.30 g, 16.0 mmol) in anhydrous THF (25 mL) was added dropwise over 5 min. After stirring at room temperature for a further 10 min, the mixture was quenched with water and transferred to a 250 mL Erlenmeyer flask, neutralized with concentrated HCl ($\sim$10 mL), diluted with dichloromethane (55 mL) and transferred to a separatory funnel. The aqueous layer was discarded and the organic layer was washed successively with water (55 mL), 10% $NaHCO_3$ (2 $\times$ 55 mL), and water (55 mL). The organic layer was dried over sodium sulfate, evaporated in vacuo and the residue triturated with anhydrous Et_2O (110 mL). The crude solid was recrystallized from EtOAc, to give **15b** (0.75 g 21%) as cream colored crystals, mp 132–134 °C ^{1}H NMR ($CDCl_3$) δ 1.0 (3H, J = 6.4 Hz, CH_3), 2.0–2.7 (8H, m, cyclohexene ring 5H, and the isoxazole ring CH_3), 6.4 (1H, s, CH=), 6.8 (1H, s (split), CH= on isoxazole ring), 8.3 (1H, br s, N-H). ^{13}C NMR ($CDCl_3$) δ 12.63, 20.94, 29.11, 36.68, 44.95, 101.46, 112.97, 152.95, 154.03, 157.31, 172.28 and 199.72. IR (KBr) 3397.41 cm^{-1} (NH stretch), 3143.68 cm^{-1} (5-methylisoxazole stretch), 1685.35 cm^{-1} and 1618.99 cm^{-1} (two C=O stretches). Anal. (C, H, N).

3.3.2. 5-Methyl-N-(3-oxocyclohex-1enyl)isoxazole-3-carboxamide (**15a**)

The above procedure was repeated using amino ketone **14a** (1.33 g, 12 mmol), to give **15a** (0.60 g, yield, 23%), as yellow crystals, mp 132–134 °C (from EtOAc) ^{1}H NMR ($CDCl_3$) δ 1.9–2.7 (6H, m, cyclohexene ring), 2.4 (3H, s, CH_3), 6.4 (1H, s, CH=), 6.8 (1H, s, (split), H on the isoxazole ring), 8.2 (1H, s, br, N-H). ^{13}C NMR ($CDCl_3$) δ 11.48, 24.32, 32.89, 42.57, 50.52, 101.45, 112.26, 151.32, 157.36, 158.23, 172.30 and 199.50. IR (KBr) 3285.07 cm^{-1} (NH stretch), 3153.70 cm^{-1} (5-methylisoxozole stretch), 1675.11 cm^{-1} and 1623.90 cm^{-1} (two C=O stretches). Anal. (C, H, N).

3.4. Trifluoromethyl analog

3.4.1. Tert-Butyl 4-(5-methylisoxazole-3-ylamino)-6-trifluoromethyl-2-oxocyclohhex-3-ene carboxylate (**19**)

To a pre-dried 2-neck 250 mL round bottom flask fitted with a condenser and magnetic stirrer was added 25 mL of anhydrous EtOH, followed by 1.09 (47.6 g, 47.6 g-atom) of sodium. Complete solution was achieved by gentle reflux. After cooling to room

temperature, tert-butyl acetoacetate, **17** (7.53 g, 47.6 mmol) was slowly added and the mixture stirred for an additional 30 min. Ethyl 4,4,4-trifluorocrotonate, **16** (8.0 g, 47.6 mmol), dissolved in 25 mL of anhydrous EtOH was added and the mixture was refluxed for 2.5 h. Evaporation under vacuum produced an orange gummy crude residue. The residue was crystallized from EtOAc to provide tert-butyl 6-(trifluoromethyl)-4-hydroxy-2-oxocyclohex-3-ene carboxylate, **18** (4.55 g, 34.1%), mp 133–137 °C ^{1}H NMR (DMSO-$_{d_6}$) δ 1.9 (9H, m, 3× CH_3), 2.6–3.5 (4H, m, cyclohexene ring), 5.4 (1H, s, CH=), 9.0 (1H, s, OH). Anal. (C, H, N, F).

To a 200 mL round bottom flask was added 50 mL of EtOAc and 50 mL of anhydrous EtOH followed by **18** (1.41 g, 5.0 mmol). After solution, 3-amino-5-methylisoxazole (0.61 g, 6.23 mmol) was added and after solution, the mixture was refluxed for 6 h. The mixture was evaporated under reduced pressure to yield a yellow oil. The crude product was recrystallized from a Et_2O/hexane solvent system to obtain the title compound, **19** (403 mg, 22.4%), mp 225–226 °C ^{1}H NMR (DMSO-$_{d_6}$) δ 1.4 (9H, m, 3× CH_3), 2.4 (3H, s, CH_3 on isoxazole ring), 2.7–3.4 (4H, m, cyclohexene ring), 6.3 (1H, s, CH=), 9.9 (1H, s, NH). Anal. (C, H, N, F).

3.5. Pharmacology

Initial evaluation for anticonvulsant activity was done by the Epilepsy Branch, National Institute of Neurological Disorders and Stroke [28]. These tests were performed in male Carworth Farms no. 1 (CF1) mice. Phase I of the evaluation included three tests: maximal electroshock (MES), subcutaneous pentylenetetrazole (scPTZ), and rotorod test for neurological toxicity (Tox). Compounds were suspended in 30% poly(ethyleneglycol) 400 and were administered by intraperitoneal injection at three dosage levels (30, 100 and 300 mg kg^{-1}) with anticonvulsant activity and neurotoxicity noted 30 min and 4 h after administration. Data is found in Table 3. Phase VIB determined the median effective dose (ED_{50}) and median toxic dose (TD_{50}) in male Sprague–Dawley rats. The ED_{50} and TD_{50} values and their confidence limits were determined at the time of peak effect for **12b** by the method of Litchfield and Wilcoxon [29]

3.6. X-ray crystal analysis

3-((5-Methylisoxazol-3-yl)amino)-5-methylcyclohex-2-anone (**12b**) and 3-((5-methylisoxazol-3-yl)amino)-5,5-dimethylcyclohex-2-enone (**12c**) were recrystallized from EtOAc. All experimental details related to the structural analysis are provided in Figs. 1 and 2, and Tables 4 and 5. The structure was solved by direct methods of the SHELXTLPC program and refined by the SHELXTL program [45]. The full crystallographic data were deposited at the Cambridge Crystallographic Data Center. Copies of the data can be obtained free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (Fax: +44 1223 336033 or email: deposit@ccdc.camac.uk). The database numbers are CCDC ID: 280424 (**12b**) and CCDC ID: 289425 (**12c**).

3.7. Log P

At pH 7.4 phosphate buffer was prepared by dissolving a premixed buffer salt (Sigma–Aldrich) in an appropriate volume of deionized water. The aqueous buffer was saturated with octanol, prior to partitioning, by adding octanol (HPLC grade), mixing, and allowing the phases to separate overnight. Octanol (HPLC grade) was saturated with the buffer in the same manner. A stock solution was prepared for each sample by dissolving a small portion (~2 mg) in 150 mL of the buffer. A 150 mL portion of the buffer was used a blank for the stock solutions. The sample solutions and blank were shaken for approximately 1 h at ambient temperature, then gravity filtered to remove any undissolved compound. Aliquots (~10 mL) of the blank and stock solutions were transferred to test tubes and centrifuged for approximately 45 min before determining the absorbance. A 100 ml portion of each stock and blank solution was volumetrically transferred to individual separatory funnels, and 5 mL of octanol was added. After the separatory funnels were gently inverted ~100 times, the phases were allowed to separate for about 1 1/2 h. Aliquots (~10 mL) of the partitioned blank and sample solutions were transferred to test tubes and centrifuged for approximately 45 min before determining the absorbance. The absorbance of the aqueous phase was determined for each solution, before and after partitioning, using a HP 8453 UV/Vis spectrophotometer and scanning from 400 to 200 nm. The appropriate blank was used to determine the baseline for each solution.

4. Conclusion

The mechanism of action of these compounds yielded no positive results for sodium channel activity. At the present time, the mode of action at which these compounds elicit their activity is unknown. Additional tests are underway to evaluate the active isoxazole analogs' ability to positively potentiate GABA and this will be reported shortly.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ejmech.2012.02.003. These data include MOL files and InChIKeys of the most important compounds described in this article.

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