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Original article

Synthesis and anticonvulsant activity of enaminones. 4. Investigations on isoxazole derivatives

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Abstract

Due to the exceptional anticonvulsant activity displayed by substituted aniline enaminones, related pyridine derivatives and phenothiazines synthesised in our laboratories, the further investigation of various aromatic heterocycles was undertaken. Condensation of cyclic 1,3-diketo esters with 3-, and 5-aminoisoxazole derivatives led to a series of potent anti-maximal electroshock (MES) analogues, three of which occurred in the 3-amino series: ethyl ester (**10**), orally (po) active in rats [ED_{50} 68.9 mg kg^{-1}, $TD_{50} > 500$ mg kg^{-1}, protective index ($PI = TD_{50}/ED_{50}$) > 49.6]; methyl ester (**9**), ED_{50} 68.9 mg kg^{-1} intraperitoneally (ip) in mice, $TD_{50} > 500$ mg kg^{-1}, $PI > 7.3$, and *tert*-butyl ester (**8**), ED_{50} 28.1 mg kg^{-1} po in rats, $TD_{50} > 500$ mg kg^{-1}, $PI > 17.8$. Sodium channel binding studies, as well as evaluations against pentylenetetrazol, bicuculline, and picrotoxin on isoxazole **10** were all negative, leading to an unknown mechanism of action. X-ray diffraction patterns of a representative of the 3-amino series (isoxazoles **6**–**11**) unequivocally display the existence of intramolecular hydrogen bonding of the nitrogen to the vinylic proton in the cyclohexene ring, providing a pseudo three ring structure which was also shown previously with the vinylic benzamides. Physicochemical-permeability across the BBB suggested an efflux mechanism for the previously synthesised aniline enaminones, but not with isoxazole **10**. © 2002 Published by Éditions scientifiques et médicales Elsevier SAS.

Keywords: Enaminones; Isoxazoles; Maximal electroshock seizure test; Anticonvulsant activity; X-ray crystallography; Structure–activity relationship; Physicochemical permeability

Abbreviations: ADD, Antiepileptic drug development; AP, apical; BBMEC, bovine brain microvessel endothelial cells; BL, basolateral; b.p., boiling point; Clog *P*, calculated log *P*; CSD, Cambridge Structural Database; ED_{50}, effective dose for 50% of test animals; EtOAc, ethyl acetate; [^{3}H]BTX-B, [^{3}H]batrachotoxinin A 20 α-benzoate; IC_{50}, 50% in vitro inhibition of binding of [^{3}H]BTX-B to sodium channels in rat brain synaptoneurosomes; ip, intraperitoneal; MDR, multidrug resistance; MES, maximal electroshock seizure; m.p., melting point; NINDS, National Institutes of Neurological Disorders and Stroke; Pgp, P glycoprotein; PI, protective index; scBic, subcutaneous bicuculline; scPic, subcutaneous picrotoxin; scPTZ, subcutaneous pentylenetetrazol; TD_{50}, toxic dose for 50% of test animals; TD_{50}/ED_{50}; THF, tetrahydrofuran; Tox, neurologic toxicity; TTE, threshold tonic extension.

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1. Introduction

The search for antiepileptic compounds with more selective activity and lower toxicity continues to be an area of investigation in medicinal chemistry [1]. Many patients with epilepsy fail to experience adequate control of their seizures, despite the optimal use of available antiepileptic drugs. Other patients do so only at the expense of significant toxic side effects [2]. In a continuing study of potential anticonvulsants [3–7], we have extended the initial evaluation of the enaminones bearing the aniline and benzylamine moieties to aromatic heterocycles, initially with the pyridine system [4], and currently the isoxazole nucleus. A preliminary report of this latter investigation has been published [8]. The study of anticonvulsant isoxazoles has been reported in a number of laboratories [1,9–11], and principally involved condensation of an acid synthon with an aminoisoxazole, effectively producing an amide. Our work, however, produces a secondary amine which, in our laboratory, has previously been shown to produce a distinctly different spectrum of anticonvulsant activity. The active benzylamines, in our hands, retained anticonvulsant activity exclusively in the maximal electroshock seizure (MES) evaluation, while the active benzamides possessed dual activity in both the electroshock and the subcutaneous pentylenetetrazol (scPTZ) models [7].

2. Chemistry

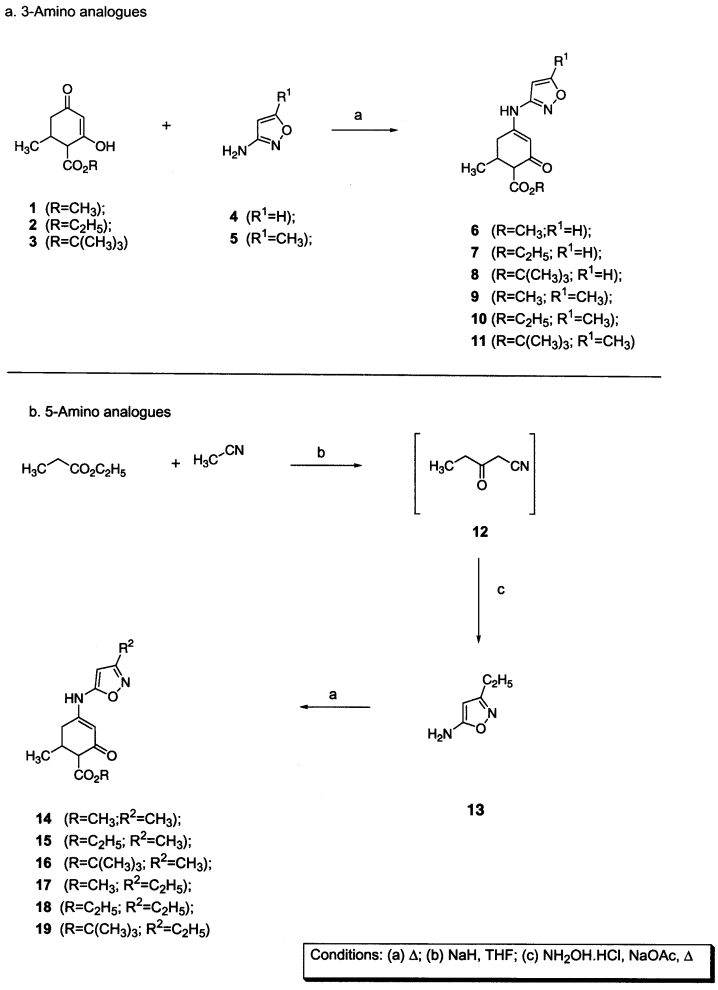
2.1. Synthesis

The synthesis of the isoxazole enaminones which appear in Table 1 is indicated in the following scheme (Scheme 1). The β-hydroxy keto esters, **1**–**3**, were prepared as previously reported [3–7] and were refluxed with the requisite 3-amino isoxazoles, **4**, and **5**, or the 5-amino isoxazoles (commercially available 3-methy-5-aminoisoxazole) or previously unreported 3-ethyl-5-aminoisoxazole (**13**), either as previously reported, or more recently, in a mixture of benzene, or toluene, and ethyl acetate (70:30) in a Dean-Stark trap, the reaction monitored by the water formation. The unreported 3-ethyl-5-aminoisoxazole (**13**), was synthesised by modification of the method of Nishiwaki and Saito [12], condensing ethyl propionate with acetonitrile in the presence of sodium hydride to form cyanoketone, **12**, which was subsequently reacted with hydroxylamine hydrochloride and sodium acetate to form 3-ethyl-5-aminoisoxazole (**13**), in reasonable yields. Condensation of 3-ethyl-5-aminoisoxazole (**13**) with the β-keto esters (**1**–**3**) under previously indicated conditions provided the requisite 3-ethyl isoxazoles (**14**–**19**). The stereochemistry of the products (**6**–**11**, and **14**–**19**) indicates that they exist as *trans* racemates, which was previously reported by Friary et al. [13] and by us earlier [14].

Table 1
Anticonvulsant screening project (ASP): anticonvulsant results

Compound	Clog P [a]	Anticonvulsant results [b]
3-*Aminoisoxazoles*		
6	0.89	phase I (mice): Class 2 (1 death at 30 mg kg^{-1} in scPTZ test). Active in the rat at po 30 mg kg^{-1} (3/4) at 2 h (0/4 toxic at all time periods at 30 mg kg^{-1})
7	1.42	phase I (mice): Class 1 (MES) (1 tonic extension at 100 mg kg^{-1} is scPTZ test)
8	2.13	phase I (mice): Class 2 (1 death at 100 mg kg^{-1} in scPTZ test)
9	1.39	phase I (mice): Class 1 (MES) Mice: ED_{50} 149.4 mg kg^{-1}; TD_{50} > 500 mg kg^{-1} (at 1 h) Rats: ED_{50} 28.1 mg kg^{-1}; TD_{50} > 500 mg kg^{-1} (at 1 h)
10	1.91	phase I (mice): Class 1 (MES) Mice: ED_{50} 68.9 mg kg^{-1}; TD_{50} > 500 mg kg^{-1} (at 0.25 h)
11	2.62	phase I (mice): Class 1 (MES) Mice: ED_{50} 119.9 mg kg^{-1}; TD_{50} > 500 mg kg^{-1} (at 0.25 h) (repeat ED_{50} 149.9 mg kg^{-1}) Rats: ED_{50} 10.1 mg kg^{-1}; TD_{50} > 500 mg kg^{-1} (at 4 h)
5-*Aminoisoxazoles*		
14	1.39	phase I (mice): Class 1 (MES). Active in the rat at po 30 mg kg^{-1} (2/4) at 0.25, 0.5 and 1 h (0/4 toxic at all time periods at 30 mg kg^{-1})
15	1.91	phase I (mice): Class 3 (MES)
16	2.62	phase I (mice): Class 1 (MES). Active in the rat at po 30 mg kg^{-1} (3/4) at 4 h (0/4 toxic at all time periods at 30 mg kg^{-1})
17	1.91	phase I (mice): Class 3 (MES)
18	2.44	phase I (mice): Class 3 (MES)
19	3.15	phase I (mice): Class 3 (MES)

[a] Calculated from reference [28].
[b] 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}.



Scheme 1.

2.2. NMR analysis

The NMR results at 300 MHz of the isoxazolyl enaminones were consistent with the assigned structures. We had previously reported [4] that in comparing methyl 4-[(4-chlorophenyl)amino]-6-methyl-2-oxocyclohex-3-ene-1-carboxylate, **20a** (Fig. 1, $R^3 = 4$-Cl) with the analogous 2-pyridine analogue (**21**), there was a difference in the vinyl proton assignment. The vinyl proton for the 4-chlorophenyl derivative (**20a**), appeared at δ 5.48 ppm, while the vinyl proton in the pyridine analogue (**21**) was deshielded and appeared at δ 6.82 ppm, indicating involvement of this proton in intramolecular hydrogen bonding. Table 2 summarises the vinyl proton assignments for the reported isoxazoles. In our previous NMR studies on the enaminones [14], we have shown that hydrogen-bonding with nitrogen or oxygen atoms attached to enaminone system display deshielding of the vinylic proton from δ values <6 to >6 ppm. This is also noted in Table 2. Anisotropy of the aromatic ring has little influence on shielding the vinylic proton of the 4-chloro enaminone (**20a**), (δ 5.48 ppm) when compared with the pyrrolidino enaminone (**22**), (δ 5.08 ppm), the cyclohexylamino enaminone (**23**) (δ 5.17 ppm), and the moropholino enaminone (**24**) (δ 5.30 ppm), all of which are devoid of aromatic protons [14]. In addition to the X-ray studies which follow, Edafiogho performed an X-ray crystallographic study on methyl 4-[(4-bromophenyl)amino]-6-methyl-2-oxocyclohex-3-ene-1-carboxylate (**20b**) (Fig. 1, $R^3 = 4$-Br) and determined that the phenyl ring is planar with the cyclohexenone ring and, as with the 4-chloro enaminone (**20a**), is not likely to be in a conformation where the vinylic proton would be above the aromatic ring, to cause a high-field shift due to anisotropy of the aromatic ring [15].

In Table 2, the agreement between the 3-aminoisoxazoles (**9**–**11**; $R^1 = CH_3$) and the comparable 5-aminoisoxazoles (**14**–**16**; $R^1 = CH_3$) is quite good and clearly shows the deshielding effect noted with the pyridine analogue (**21**). This deshielding effect was most marked with the unsubstituted 3-aminoisoxazoles (**9**–**11**; $R^1 = H$) and was significantly diminished with the ethyl analogues (**17**–**19**; $R^1 = C_2H_5$). This latter effect

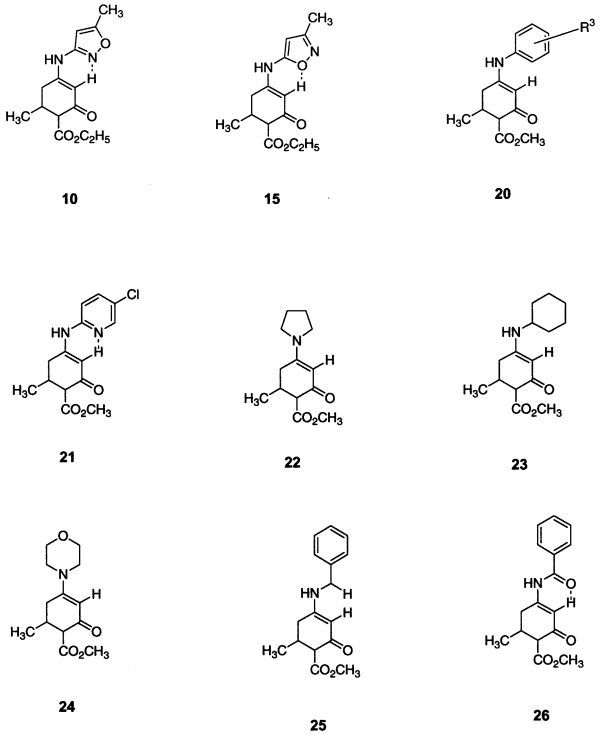


Fig. 1. NMR structural correlation for vinylic coupling in compounds **20a** (R^3 = 4-Cl), **21** [4,14], **22**–**24** [14], and **23** [7]. X-ray analysis of **10** is shown in Fig. 2 and of **21** in Fig. 3.

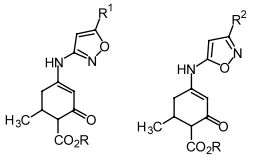
could be correlated to the uniform inactivity of this latter series. Recently [7], we showed that the vinyl deshielding effect also occurred when the benzylamines (**25**), were compared with the analogous benzamides, (**26**). In the latter series, the hydrogen bonding occurred between the carbonyl oxygen and the vinylic proton, whereas, intramolecular hydrogen bonding did not occur with the benzylamines (**25**). This pseudo three ring phenomenon was further verified by X-ray diffraction analysis of two benzamides [7]. Thus, in this latter study, we were able to provide a direct correlation between the NMR analysis and the X-ray crystal structure.

2.3. X-ray crystallography

To further define the role of three dimensional structure to anticonvulsant activity, an X-ray diffraction study of a representative 3-aminoisoxazole (**10**) was performed as well as that of the 2-amino-5-chloropyridine analogue (**21**). As noted in Fig. 2 of the isoxazole compound (**10**) and in Fig. 3 of the 5-chloropyridine analogue (**21**), strong hydrogen-bonding occurred between the vinyl protons and the nitrogen atoms. This has been seen previously with respect to earlier structures [6,7]. In either case, the pseudo three ring configuration was unequivocally shown. Tables 3 and 4 provide the experimental data detailing the crystal data, data collection, and refinement data for isoxazole **10** and the pyridine analogue **21**, respectively. Additionally, Table 5A and Table 6A provide selected torsion angles which illustrate the pseudo three-ring configuration in isoxazole **10** and the pyridine compound **21**, respectively. Molecule A and Molecule B are the enantiomeric forms of each compound, and Table 5B and Table 6B provide the most important hydrogen bonds supporting the hypothesis for isoxazole **10** and pyridine compound **21**, respectively. While X-ray crystal data on our enaminones are modest, the hydrogen bonding ensures the rings are planar. It is apparent that planarity of the molecules is an essential factor in the determination of biological activity, because as shown in the NMR studies for that non-planar pyrrolidino analogue **22**, the cyclohexylamino analogue **23** and the morpholino compound **24** have been shown to be inactive in our hands.

As further justification of the pseudo three ring hypothesis, a search of the Cambridge Structural Database (CSD) [16] for potential intermolecular or intramolecular hydrogen bonding interactions between

Table 2
Vinyl proton assignments for the isoxazole enaminones



Compound	R	R^1	δ, ppm	Compound	R	R^2	δ, ppm
6	CH_3	H	6.39	**14**	CH_3	CH_3	6.03
7	C_2H_5	H	6.38	**15**	C_2H_5	CH_3	6.14
8	$C(CH_3)_3$	H	6.31	**16**	$C(CH_3)_3$	CH_3	6.10
9	CH_3	CH_3	6.02	**17**	CH_3	C_2H_5	5.54
10	C_2H_5	CH_3	6.02	**18**	C_2H_5	C_2H_5	5.54
11	$C(CH_3)_3$	CH_3	6.01	**19**	$C(CH_3)_3$	C_2H_5	5.54

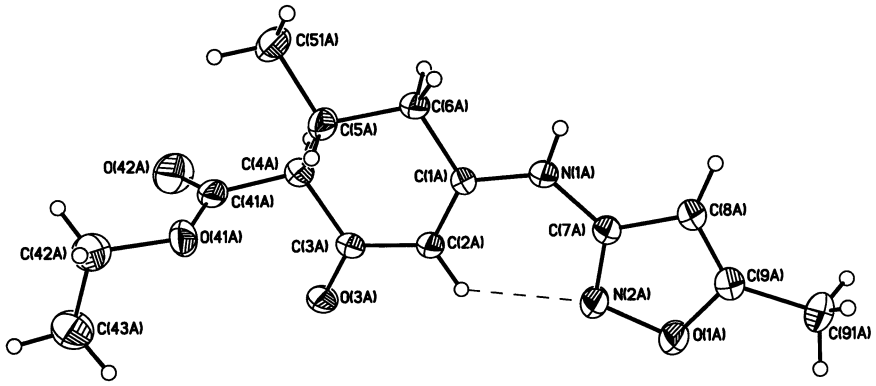


Fig. 2. X-ray crystal structure of ethyl 4-[(5-methyl-3-isooxazoly)amino]-6-methyl-2-oxo-3-cyclohexane-1-carboxyalte (**10**). Note dotted lines indicating the N···H bonding interaction.

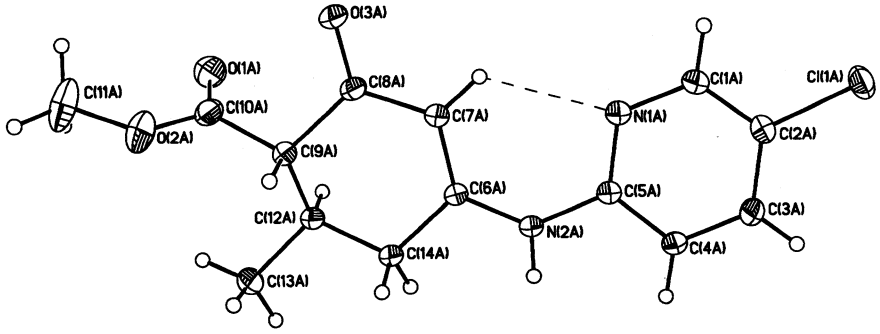


Fig. 3. X-ray crystal structure of methyl 4-[(5-chloro-2-amino)pyridyl]-6-methyl-2-oxo-3-cyclohexene-1-carboxylate (**21**). Note dotted lines indicating the N···H bonding interaction.

vinyl protons as donors and oxygen atoms (selecting a screening H···O distance between 1.8 and 2.5 Å) as acceptors gave a total of 11 241 hits. While some of these interactions could be discarded as not being hydrogen-bonding in character based on their small C–H···O angle, nevertheless the mean H···O distance was 2.448 Å. The distribution of C–H···O angles were bimodal with a minor cluster centred between 95 and 100° and a main cluster centred between 155 and 160°. These results clearly show that a hydrogen-bonding interaction between a vinylic proton and oxygen is a relatively common occurrence in the solid state. An additional search of CSD found the following information regarding –C=C–H···N interactions: (a) intramolecular bonding-195 hits; average –C=C– bond, 1.348 Å; average –C–H bond, 0.993 Å; average H···N distance, 2.410 Å; average –C=C–H bond angle, 119.916°; average –C–H···N bond angle, 111.413°; while (b) intermolecular bonding, 262 hits; average –C=C– bond, 1.348 Å; average –C–H bond, 1.0212 Å; average H···N distance, 2.420 Å; average –C=C–H bond angle, 123.030°; average –C–H···N bond angle, 154.125°. In the case of intramolecular interactions, the C–H···N angle would be expected to be constrained to a much smaller degree than would be the case where there no such constraint existed (i.e. the C–H···N angles of 111.413 and 154.125° for the intra- and intermolecular interactions, respectively). In all of the other parameters, the average values agree very well between the two different situations. While the CSD search was global, it is acknowledged that the protons of the enamines represent a small percentage of the total search. Nevertheless, it is feasible to assume that the vinylic protons of the enamines play a role in hydrogen-bonding. Our argument would have been conclusive if the X-ray structure of the inactive isomeric isoxazole (Fig. 1, **15**) could be determined, however, single crystals of the compound could not be grown in sufficient size to afford a definitive analysis. X-ray structural data for both molecules, **10** and **21**, have been sent to the Cambridge Crystallographic Data Centre and are designated CCDC 182110 and CCDC 182109 respectively.

3. Pharmacology

3.1. Preliminary pharmacological testing

The Antiepileptic Drug Development (ADD) Program, Epilepsy Branch, Neurological Disorders Program, National Institute of Neurological Disorders and Stroke (NINDS) have provided preliminary pharmaco-

logical testing of the reported compounds. These testing procedures have been described [2,17,18]. Phase I studies of the isoxazoles involved three tests: MES, scPTZ, and neurologic toxicity (Tox). Intraperitoneal (ip) administration of the test compounds was as a suspension in 0.5% methylcellulose. As previously reported [4–7], active compounds in the Phase I evaluation were subsequently tested either for an ED_{50} quantitation in mice (Phase II), or qualitatively in rats (Phase VIA). If successful in Phase VI, rat quantitation (Phase VIB) was undertaken. These data are provided in Table 1. An initial screen of starting 3-amino-5-methyisoxazole (**3**) indicated that it was inactive in the standard Phase I evaluations as well as in the threshold tonic extension (TTE) test [19].

3.2. Sodium channel binding studies

We had previously evaluated methyl 4-[(4-chlorophenyl)amino]-6-methyl-2-oxocyclohex-3-ene-1-carboxylate (**20a**) (R^3 = 4-Cl) and methyl 4-[(4-trifluoromethoxyphenyl)amino]-6-methyl-2-oxocyclohex-3-ene-1-carboxylate (**20c**) (R^3 = 4-OCF_3) as inhibitors of the specific binding of [^{3}H]batrachotoxinin A 20 α-benzoate to neurotoxin site 2 of the voltage-dependent sodium channel [5]. The IC_{50} data indicated that both were sodium channel inhibitors (the 4-chloro enaminone (**20a**), at 489 μM; the 4-trifluoromethoxy enaminone (**20c**), at 170 μM). In view of the similarity in their initial pharmacological results, the ethyl ester of the 3-amino series (**10**), was evaluated in this assay. It was, however, found to be inactive under the test conditions [20,21].

Table 3
Crystal data and structure refinement for ethyl 4-[(5-methyl-3-isoxazolyl)-amino)]-6-methyl-2-oxo-3-cyclohexene-1-carboxylate (**10**)

Parameter	
Identification code	**10**
Empirical formula	$C_{14}H_{18}N_2O_4$
Formula weight	278.30
Temperature (K)	293 (± 2)
Wavelength	0.71073
Crystal system	triclinic
Space group	*P*1
Unit cell dimensions	
a (Å)	6.839 (3)
b (Å)	11.619 (5)
c (Å)	19.513 (7)
α (°)	106.70 (11)
β (°)	91.64 (11)
γ (°)	98.80 (10)
Volume, Z (Å^3)	1463.2 (10)
Z	4
Density (calculated) (mg m^{-3})	1.259
Absorption coefficient (mm^{-1})	0.093
F(000)	592
Crystal size (mm)	0.07 × 0.79 × 0.23
θ Range for data collection (°)	3.02–24.91
Reflections collected	3873
Independent reflections	3502 (R_{int} = 0.0225)
Completeness to θ = 24.91°	68.7%
Absorption correction	None
Refinement method	Full-matrix least-squares on F^2
Data/restraints/parameters	3502/10/449
Goodness-of-fit on F^2	1.031
Final *R* indices [$I>2\sigma(I)$]	R_1 = 0.0617, wR_2 = 0.1336
R indices (all data)	R_1 = 0.1080, wR_2 = 0.1579
Extinction coefficient	0.0034 (13)
Largest diffraction peak and hole (e Å^{-3})	0.421 and −0.213

Table 4
Crystal data and structure refinement for methyl 4-[(5-chloro-2-amino)pyridyl]-6-methyl-2-oxo-3-cyclohexene-1-carboxylate (**21**)

Parameter	
Identification code	**21**
Empirical formula	$C_{14}H_{15}ClN_2O_3$
Formula weight	294.73
Temperature (K)	293 (± 2)
Wavelength	0.71073
Crystal system	triclinic
Space group	*P*1
Unit cell dimensions	
a (Å)	6.6451 (10)
b (Å)	11.9653 (16)
c (Å)	18.984 (2)
α (°)	107.585 (11)
β (°)	91.289 (11)
γ (°)	96.323 (10)
Volume (Å^3)	1427.6 (3)
Z	4
Density (calculated) (mg m^{-3})	1.371
Absorption coefficient (mm^{-1})	0.276
F(000)	616
Crystal size (mm)	0.10 × 0.80 × 0.64
θ Range for data collection (°)	2.25–27.50
Reflections collected	6548
Independent reflections	6007 (R_{int} = 0.0179)
Completeness to θ = 27.50°	91.3%
Absorption correction	SHELXA
Maximum and minimum transmission	0.8932 and 0.6365
Refinement method	full-matrix least-squares on F^2
Data/restraints/parameters	6007/8/435
Goodness-of-fit on F^2	1.016
Final *R* indices [$I>2\sigma(I)$]	R_1 = 0.0456, wR_2 = 0.1111
R indices (all data)	R_1 = 0.0683, wR_2 = 0.1243
Extinction coefficient	0.0036 (10)
Largest diffraction peak and hole (e Å^{-3})	0.293 and −0.266

Table 5
Selected torsion angles for ethyl 4-[(5-methyl-3-isoxazolyl)-amino)]-6-methyl-2-oxo-3-cyclohexene-1-carboxylate, (**10**) and methyl 4-[(5-chloro-2-amino)pyridyl]-6-methyl-2-oxo-3-cyclohexene-1-carboxylate (**21**)

(*A*)	
Molecule A	
H(2AA) C(2A) C(1A) N(1A)	3.0
C(1A) N(1A) C(7A) N(2A)	−2.8
Molecule B	
H(2BA) C(2B) C(1B) N(1B)	3.1
C(1B) N(1B) C(7B) N(2B)	−4.1
(*B*)	
Molecule A	
H(7AA) C(7A) C(6A) N(2A)	2.5
C(6A) N(2A) C(5A) N(1A)	−2.1
Molecule B	
H(7BA) C(7B) C(6B) N(2B)	2.8
C(6B) N(2B) C(5B) N(1B)	−8.4

Table 6
Selected torsion angles for ethyl 4-[(5-methyl-3-isoxazolyl)-amino)]-6-methyl-2-oxo-3-cyclohexene-1-carboxylate (**10**) and methyl 4-[(5-chloro-2-amino)pyridyl]-6-methyl-2-oxo-3-cyclohexene-1-carboxylate (**21**)

C–H (Å)	H···N (Å)	C–H···N (°)	C···N (Å)
(*A*)			
Molecule A			
0.930	2.277	125.1	2.914
Molecule B			
0.930	2.317	124.9	2.949
(*B*)			
Molecule A			
0.930	2.326	123.1	2.939
Molecule B			
0.930	2.295	123.3	2.912

study, compared the 4-chloro prototype (**20a**) with the ethyl ester of the 3-amino series (**10**), in the in vitro primary cell culture model consisting of bovine brain microvessel endothelial cells (BBMEC) [24–27]. This data is summarised in Table 7. As noted, Clog *P* [28], chromatographic retention data (expressed as log K_{IAM}), apical (AP) to basolateral (BL) transport as well as BL to AP were significantly different for these active anticonvulsant compounds. These data suggests that for the 4-chloro enaminone (**20a**), and other anilino enaminones [22], their transport was active and not passive and that there was an efflux mechanism (e.g. Pgp or MRP) responsible for the high BL to AP transport of these analogues. This efflux mechanism, however, did not effect the isoxazole (**10**). While these data explain the extended (ca. 4 h) protection of the isoxazole (**10**), but does not explain how it does, in fact, exerts its anticonvulsant effect.

4. Results and discussion

Pharmacological comparison of the isomeric 5-methyl-substituted isoxazoles (**9**–**11**) with the 3-methyl-substituted isoxazoles (**14**–**16**) in Table 1 reveal both similarities and differences. Both methyl esters, (**9**) and (**14**), were MES-active in both rodent species, while only the ethyl ester of the 3-amino series (**10**), was active. The *tert*-butyl ester in the 3-amino series (**11**) gave irreproducible results in mice, but excellent results in rats, with the best PI in that series of all analogues tested, while the comparable 5-amino analogue (**16**), also produced an active compound in both species. The presence or absence of the alkyl substitution, R^1, on the isoxazole ring, can further differentiate the 3-amino series. Clearly, the anti-MES activity for unsubstituted isoxazoles (**6**–**8**) was comparable to the 5-methyl-substituted isoxazoles (**14**–**16**), however, the unsubstituted series resulted in highly toxic compounds in the scPTZ evaluation. As noted in Table 1, deaths occurred with isoxazoles (**6**) and (**8**) under standard assay conditions. In the 5-amino series, extension of the alkyl substitution, producing 3-ethyl analogues (**17**–**19**), resulted in inactive compounds.

The mechanism of action of the isoxazoles in this study remains to be determined. As expected from

Table 7
Chemical, pharmacological and permeability (P_{app}) properties of isoxazole **10** and enaminone **20a** (R^3 = 4-Cl) [a]

Compound	ED_{50} (rat, po, mg kg^{-1})	Clog *P* [b]	log K_{IAM}	P_{app}		
				AP → BL [c]	BL → AP	BL → AP/AP → BL
10	10.1	1.91	−1.60	23.47 (±0.72) [d]	18.41 (±0.32)	0.78
20a	5.9	2.98	0.11	8.33 (±0.43)	19.63 (±0.54)	2.36

[a] Calculated from reference [23].
[b] Calculated from reference [28].
[c] P_{app} coefficients listed as 10^{-5} cm s^{-1}.
[d] Data listed as the mean at $n = 3$ (± S.D.).

Table 8
Prelimiary hippocampal kindling screen in rats, ip, of isoxazole **11** [a]

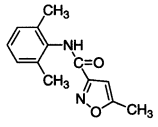
	Seizure score, pre-drug	Seizure score, drug	After discharge duration (s), pre-drug	After discharge duration (s), drug
Rat #1	5	3	58–71	36
Rat #2	5	4	51–79	83

[a] Dose = 300 mg kg^{-1}; time of maximum effect = 45 min.

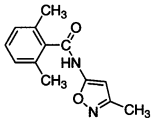
previous studies with the enaminones [3–7], Phase I test against pentylenetetrazol was consistently negative. The compounds were subsequently evaluated against bicuculline and picrotoxin, compounds which delineate whether the test substance acts through GABA inhibitory function, or chloride channel inhibition, respectively. The 3-aminoisoxazole compounds (**9**, **10** and **11**) were all uniformly inactive in the subcutaneous bicuculline (scBic) and the subcutaneous picrotoxin (scPic) evaluation. Compound **9** produced death following continuous seizures in the animals at 250 and 500 mg kg^{-1} of test dose in the scBic test; isoxazole **10** produced death following continuous seizure in the animals at 125 mg kg^{-1} in the same test; while the *tert*-butyl isoxazole (**11**), did not produce any fatalities, but was inactive in both tests in doses up to 500 mg kg^{-1}. Compound **11** was evaluated in isolated hippocampal slices [29,30]. This method has the advantage of recording important electrical activity under a variety of experimental conditions and tissue penetration by the drug. However, this evaluation has the disadvantages of cell viability and the lack of interactions with the neural circuits found in the intact animal [31]. The hippocampal results on *tert*-butyl isoxazole (**11**) are shown in Table 8 and suggested only a weak ability to block the expression of fully kindled seizures. However, further testing would be required before any definitive conclusion regarding efficacy against focal seizures could be drawn. In addition, isoxazole (**11**) was evaluated for ip toxicity in rats and was completely non-toxic at dosages up to 300 mg kg^{-1} for periods up to 4 h.

The activity–inactivity of the 3-amino and 5-amino isoxazoles may be due to orientation of the pseudo three ring system.

Our synthetic approach was focused on alkyl substitution on the isoxazole ring and its effect on anticonvulsant activity. Lepage and co-workers [1] reported on the anticonvulsant activity of the isoxazole amides, from which emerged two promising candidates, *N*-(2,6-dimethylphenyl)-5-methyl-3-isoxazolecarboxamide (**28**), and *N*-(3-heremethyl-5-isoxazolyl)-2,6-dimethylbenzamide (**29**). These isosteres provided MES protection at po rat ED_{50} 5.5 mg kg^{-1} and $TD_{50} > 500$ mg kg^{-1} for carboxamide (**28**) and an ED_{50} of 8.9 mg kg^{-1} and a $TD_{50} > 500$ mg kg^{-1} for benzamide (**29**), and similarly to our series, displayed greater sensitivity in the rat than the mouse. As noted in Table 1, Clog *P* [28] measurements of the reported isoxazoles varied from 0.89 to 3.68, while the most active compound was the *tert*-butyl ester of the 3-aminoisxazole series (**11**), being 2.62, while the other 3-methylisoxazoles, the methyl ester (**9**) and the ethyl ester (**10**), were 1.39 and 1.91, respectively. These data suggest that these compounds may be taken into the brain via the same mechanism. The Clog *P* [28] measurements for the active amides in Lepage's study are 1.38 for compound **28** and 1.88 for compound **29**, respectively. These latter values agree well with our active compounds. Hansch has indicated that the maximum potency of drugs acting on the central nervous system was obtained with congeners having an optimum lipophilicity (log P_0) near 2.0 [32]. This factor was clearly evident in the time of peak effect of our reported analogues (Table 1), as the most lipophilic compound, the *tert*-butyl isoxazole (**11**), was active at 4 h.



28



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5. Conclusions

The anticonvulsant analysis of the series of enaminone-derived isoxazoles provide a potent, orally active class of compounds with a hitherto distinct mechanism of activity which, as yet, is undefined. Their effectiveness in the MES evaluation and extended duration of action warrants further study.

6. Experimental

6.1. Chemistry

Melting points (m.p.) were determined on a Thomas–Hoover capillary m.p. apparatus and are

uncorrected. Observed boiling points (b.p.) were also uncorrected. IR spectra were recorded in Nujol, as diluted chloroform solutions in matched sodium chloride cells, or neat with a Perkin–Elmer 1330 spectrophotometer. ^{1}H-NMR spectra were recorded on a General Electric QE 300-MHz spectrometer in deuterated solvents using tetramethylsilane as an internal reference. Coupling patterns are described as follows: s, singlet; bs, broad singlet; d, doublet; dd, doubled doublet; t, triplet; q, quartet; m, multiplet and 1H, 2H, 3H, etc. as the number of hydrogens integrated within a given coupling pattern. The chemical shifts were measured to two decimal points, while the coupling constants were rounded off to one decimal place. TLC analysis employed ethyl acetate:cyclohexane (3:1) elution solvent mixture and 5×10-cm fluorescent plates (Whatman silica-gel 60A). Column chromatography employed silica gel (100–200 mesh, Fisher Scientific) and eluted with ethyl acetate:cyclohexane (3:1). Elemental analyses (C, H, and N) were performed by Schwarzkopf Microanalytical Laboratory, Woodside, NY 11377 USA. The analytical results for the elements were within $\pm 0.4\%$ of the theoretical values. X-ray crystal analysis was performed on a Nicolet P4 diffractometer. Methyl 6-methyl-2,4-dioxocyclohexane-1-carboxylate, (**1**) ($R = CH_3$) [3], ethyl 6-methyl-2,4-dioxocyclohexane-1-carboxylate (**2**) ($R = C_2H_5$) [3], and *tert*-butyl 6-methyl-2,4-dioxocyclohexane-1-carboxylate (**3**) ($R = C(CH_3)_3$) [13], were prepared by literature methods. 3-Aminoisoxazole (**4**) ($R^1 = H$), and 5-amino-3-methylisoxazole were obtained from Aldrich Chemical Company and 3-amino-5-methylisoxazole (**5**) ($R^1 = CH_3$), was obtained from Fluka Chemika AG (Germany) and used without further purification.

6.2. General procedure for the preparation of 3-substituted 5-aminoisoxazoles

6.2.1. 3-Ethyl-5-aminoisoxazole (*13*)

The procedure of Nishiwaki and Saito [12] was modified as follows. Into a 500 mL single neck flask equipped with a Y tube, a condenser, a 125 mL pressure equalising dropping funnel and a magnetic stirring bar was introduced 100 mL dry THF under nitrogen. Sodium hydride (9 g of a 50% suspension in mineral oil, 0.19 mol), and acetonitrile (7.0 g, 0.17 mol) in 20 mL of dry THF were slowly added maintaining reflux conditions. The mixture was refluxed for a total of 4 h after addition and was allowed to stir at room temperature (r.t.) for 8 h. The reaction was quenched with 200 mL of ether and the solid obtained was filtered and air dried. The residue from four reactions were combined and dissolved in a minimum volume of water. Acidification by the dropwise addition of HCl yielded an oil that was extracted with ether (4×100 mL) and dried over anhydrous sodium sulphate. Evaporation under reduced pressure yielded a yellow–brown oil which distilled at 109–110 °C (100 mm) and proved to be the crude cyanomethylethyl ketone (**12**), 16.6 g (25%); ^{1}H-NMR ($CDCl_3$): δ 1.00 (3H, t, $J = 7.0$ Hz, CH_3), 2.60 (2H, t, CH_2–C=O), 3.35 (2H, s, CH_2–CN). To a solution of sodium acetate (29.4 g, 0.36 mol) and hydroxylamine hydrochloride (22.1 g, 0.32 mol) in 200 mL of water was added the above ketone (7.49 g, 0.07 mol), dissolved in 200 mL of ethanol, added over 30 min and the mixture stirred and refluxed for 1.5 h. The alcohol was removed under reduced pressure and the sodium chloride removed and concentration repeated until a crude residue, m.p. < 100 °C resulted. Recrystallisation from methanol–petroleum ether (b.p. 60–80 °C) produced a product, **13**; m.p. 92–96 °C, 4.0 g (50%). ^{1}H-NMR ($CDCl_3$): δ 1.23 (3H, t, $J = 7.0$ Hz, CH_3), 2.59 (2H, q, $J = 7.0$ Hz, CH_2), 6.85 (1H, s, CH), 7.30 (2H, s, NH_2). Calc. for ($C_5H_8N_2O$) C, H, N.

6.3. General procedure for the preparation of 3-isoxazolylamino enaminones

6.3.1. Method A

6.3.1.1. Methyl 4-[(5-methyl-3-isoxazolyl)amino]-6-methyl-2-oxo-3-cyclohexene-1-carboxylate, (9). A mixture of methyl 6-methyl-2,4-dioxocyclohexane-1-carboxylate (**1**) (5.0 g, 27 mmol), and 3-amino-5-methyi*soxazole* (**5**) (3.24 g, 33 mmol), was added to a mixture of absolute ethanol (100 mL) and ethyl acetate (100 mL) and the solution refluxed and stirred for 6 h. Evaporation under reduced pressure yielded a yellow solid which was recrystallised three times from EtOAc to yield isoxazole **9**, 4.31 g (58%) of white crystals; m.p. 220–222 °C. ^{1}H-NMR (DMSO d_6): δ 0.98 (3H, d, $J = 5.9$ Hz, CH_3 on C_6), 2.36 (3H, s, CH_3 on isoxazole ring), 2.38–2.48 (3H, m, $CH_2 + CH$ of cyclohexene ring), 3.14 (1H, d, $J = 11.6$ Hz, CH on C_1), 3.64 (3H, s, OCH_3), 6.02 (1H, s, =CH), 6.24 (1H, s, CH on isoxazole ring), 9.64 (1H, bs, NH). Anal. ($C_{13}H_{16}N_2O_4$) C, H, N.

6.3.1.2. Ethyl 4-[(5-methyl-3-isoxazolyl)amino]-6-methyl-2-oxo-3-cyclohexene-1-carboxylate, (10). In a similar procedure, ethyl 6-methyl-2,4dioxocyclohexane-1-carboxylate (**2**), and 3-amino-5-methyiisoxazole (**5**), produced isoxazole **10**. Three recrystallisations from ethanol provided an analytical sample: yield 5.68 g (76%) of white crystals; m.p. 195–197 °C. ^{1}H-NMR (DMSO d_6): δ 1.00 (3H, d, $J = 5.7$ Hz, CH_3 on C_6), 1.20 (3H, t, $J = 7.1$ Hz, CH_3 of ethyl group), 2.35 (3H, s, CH_3 on isoxazole ring), 2.38–2.55 (3H, m, $CH_2 +$ CH of cyclohexene ring), 3.09 (1H, d, $J = 11.0$ Hz, CH on C_1), 4.12 (2H, q, $J = 7.1$ Hz, CH_2 of ethyl group), 6.02 (1H, s, =CH), 6.25 (1H, s, CH on isoxazole ring), 9.63 (1H, bs, NH). Anal. ($C_{14}H_{18}N_2O_4$) C, H, N.

*6.3.1.3. Tert-butyl 4-[(5-methyl-3-isoxazolyl)amino]-6-methyl-2-oxo-3-cyclohexene-1-carboxylate, **(11)**.* Similarly, *tert*-butyl 6-methyl-2,4-dioxocyclohexane carboxylate (**3**), and 3-amino-5-methylisoxazole (**5**), produced isoxazole **11**, as white crystals after three recrystallisations from EtOAc; m.p. 197–201 °C (with effervescence); yield, 3.57 g (43%). ^{1}H-NMR (DMSO d_6): δ 1.00 (3H, d, $J = 5.7$ Hz, CH_3 on C_6), 1.42 (9H, s, $3 \times CH_3$), 2.35 (3H, s, CH_3 on isoxazole ring), 2.44 (2H, m, CH_2 of cyclohexene ring), 2.51 (1H, m, CH of cyclohexene ring), 2.93 (1H, d, $J = 11.0$ Hz, CH on C_1), 6.01 (1H, s, =CH), 6.23 (1H, s, CH on isoxazole ring), 9.58 (1H, bs, NH). Anal. ($C_{16}H_{22}N_2O_4$) C, H, N.

*6.3.1.4. Methyl 4-[(3-isoxazolyl)amino]-6-methyl-2-oxo-3-cyclohexene-1-carboxylate, **(6)**.* In a similar procedure, ester **1** and 3-aminoisoxazole (**4**), produced isoxazole **6**. Three recrystallisations from EtOAc provided an analytical sample: yield 3.44 g (51%) of white crystals; m.p. 203–205.5 °C. ^{1}NMR indicated the compound existed as a diastereomeric mixture in a ratio of 87:13 mol%. The major isomer gave the following ^{1}H-NMR (DMSO d_6): δ 1.08 (3H, d, $J = 6.8$ Hz, CH_3 on C_6), 2.38 (1H, dd, one of $CHCH_2$), 2.50–2.70 (2H, complex m, other CH_2 + CH of cyclohexene ring), 3.05 (1H, d, $J = 11.6$ Hz, CH on C_1), 3.74 (3H, s, OCH_3), 6.28 (1H, d, $J = 1.6$ Hz, CH on isoxazole ring at C_4), 6.39 (1H, s, =CH), 8.36 (1H, d, $J = 1.6$ Hz, CH on isoxazole ring at C_3), 9.52 (1H, bs, NH). Anal. ($C_{12}H_{14}N_2O_4$) C, H, N.

*6.3.1.5. Ethyl 4-[(3-isoxazolyl)amino]-6-methyl-2-oxo-3-cyclohexene-1-carboxylate, **(7)**.* Compound **7**, was prepared from ester **2** and 3-aminoisoxazole (**4**), and occurred as white crystals after three recrystallisations from EtOAc; m.p. 180–182 °C; yield, 4.10 g (58%). ^{1}H-NMR indicated the compound existed as a diastereomeric mixture in a ratio of 89:11 mol%. The major isomer gave the following ^{1}H-NMR (DMSO d_6): δ 1.08 (3H, d, $J = 6.4$ Hz, CH_3 on C_6), 1.26 (3H, t, CH_3 of ethyl group), 2.42 (1H, dd, one of $CHCH_2$), 2.60 (2H, complex m, other CH_2 + CH of cyclohexene ring), 3.00 (1H, d, $J = 11.6$ Hz, CH on C_1), 4.18 (2H, q, CH_2 of ethyl group), 6.26 (1H, d, $J = 1.6$ Hz, CH on isoxazole ring at C_4), 6.38 (1H, s, =CH), 8.41 (1H, d, $J = 1.6$ Hz, CH on isoxazole ring at C_3), 9.54 (1H, bs, NH). Anal. ($C_{13}H_{16}N_2O_4$) C, H, N.

*6.3.1.6. Tert-butyl 4-[(3-isoxazolyl)amino]-6-methyl-2-oxo-3-cyclohexene-1-carboxylate, **(8)**.* Similarly, *tert*-butyl 6-methyl-2,4-dioxocyclohexane carboxylate (**3**) and 3-aminoisoxazole (**4**), produced isoxazole **8** as white crystals after five recrystallisations from EtOAc; m.p. 190–193 °C; yield, 4.67 g (58%). ^{1}H-NMR (DMSO d_6): δ 1.00 (3H, d, $J = 5.7$ Hz, CH_3 on C_6), 1.44 (9H, s, $3 \times CH_3$), 2.44 (2H, m, CH_2 of cyclohexene ring), 2.51 (1H, m, CH of cyclohexene ring), 2.93 (1H, d, $J = 11.0$ Hz, CH on C_1), 6.31 (1H, s, =CH), 6.27 (1H, d, $J = 1.6$ Hz, CH on isoxazole ring at C_4), 8.41 (1H, d, $J = 1.6$ Hz, CH on isoxazole ring at C_3), 9.60 (1H, bs, NH). Anal. ($C_{15}H_{20}N_2O_4$) C, H, N.

6.4. General procedure for the preparation of 5-isoxazolylamino enaminones

6.4.1. Method B

*6.4.1.1. Methyl 4-[(3-methyl-5-isoxazolyl)amino]-6-methyl-2-oxo-3-cyclohexene-1-carboxylate, **(14)**.* A mixture of ketone **1** (5.0 g, 27 mmol), and 5-amino-3-methylisoxazole (3.24 g, 33 mmol), was added to a mixture of benzene (50 mL) and ethyl acetate (15 mL) in a 100 mL single neck flask connected to a Dean-Stark trap and the mixture stirred and refluxed until the evolution of water ceased (ca. 3 h). The mixture was concentrated to ca. 1/2 its volume and allowed to stand overnight at r.t. Crystals formed which resisted recrystallisation with EtOAc and remained as a gel. Removal of the solvent under reduced pressure yielded an amorphous product that was dissolved in chloroform and eluted through a silica-gel G column (100–200 mesh) with ethyl acetate:cyclohexane (3:1). Recrystallisation of the individual 50 mL aliquots with EtOAc resulted in an analytical sample of isoxazole **14**, m.p. 189–190 °C; yield, 3.95 g (55%) as light yellow plates. ^{1}H-NMR (DMSO d_6): δ 1.08 (3H, d, $J = 6.0$ Hz, CH_3 on C_6), 2.36 (3H, s, CH_3 on isoxazole ring), 2.74 (3H, complex m, CH_2 + CH of cyclohexene ring), 3.25 (1H, d, $J =$ 11.0 Hz, CH on C_1), 3.81 (3H, s, OCH_3), 6.03 (1H, s, =CH), 7.40 (1H, s, CH on isoxazole ring), 8.11 (1H, bs, NH). Anal. ($C_{13}H_{16}N_2O_4$) C, H, N.

*6.4.1.2. Ethyl 4-[(3-methyl-5-isoxazolyl)amino]-6-methyl-2-oxo-3-cyclohexene-1-carboxylate, **(15)**.* In like manner, ester **2** and 5-amino-3-methyisoxazole provided isoxazole (**15**), m.p. 137 °C (dampens), 140–142 °C; yield 42% (after chromatography and three recrystallisations from EtOAc) as yellow crystals. ^{1}H-NMR (DMSO d_6): δ 1.09 (3H, d, $J = 6.0$ Hz, CH_3 on C_6), 1.28 (3H, t, $J = 7.0$ Hz, CH_3 of ethyl group), 2.36 (3H, s, CH_3 on isoxazole ring), 2.74 (3H, complex m, CH_2 + CH of cyclohexene ring), 3.25 (1H, d, $J = 11.0$ Hz, CH on C_1), 4.20 (2H, q, $J = 7.0$ Hz, CH_2 of ethyl group), 6.14 (1H, s, =CH), 7.44 (1H, s, CH on isoxazole ring), 8.20 (1H, bs, NH). Anal. ($C_{14}H_{18}N_2O_4$) C, H, N.

*6.4.1.3. Tert-butyl 4-[(3-methyl-5-isoxazolyl)amino]-6-methyl-2-oxo-3-cyclohexene-1-carboxylate, **(16)**.* Similarly, isoxazole **16** was prepared from ester **3** and 5-amino-3-methyisoxazole, m.p. 127–130 °C; yield 33% (after chromatography and three recrystallisations from EtOAc) as a yellow amorphous powder. ^{1}H-NMR

(DMSO d_6): δ 1.08 (3H, d, $J = 5.7$ Hz, CH_3 on C_6), 1.40 (9H, s, $3 \times CH_3$), 2.35 (3H, s, CH_3 on isoxazole ring), 2.42 (2H, m, CH_2 of cyclohexene ring), 2.55 (1H, m, CH of cyclohexene ring), 2.94 (1H, d, $J = 11.0$ Hz, CH on C_1), 6.10 (1H, s, =CH), 6.26 (1H, s, CH on isoxazole ring), 9.80 (1H, bs, NH). Anal. ($C_{16}H_{22}N_2O_4$) C, H, N.

*6.4.1.4. Methyl 4-[(3-ethyl-5-isoxazolyl)amino]-6-methyl-2-oxo-3-cyclohexene-1-carboxylate, (**17**).* A mixture of **1** (5.0 g, 27 mmol) and 5-amino-3-ethylisoxazole (**13**) (3.70 g, 33 mmol), provided isoxazole **17**, m.p. 112–113 °C; yield 15% (after chromatography and two recrystallisations from 2-propanol) as white powder. ^{1}H-NMR (DMSO d_6): δ 1.06 (3H, d, $J = 6.0$ Hz, CH_3 on C_6), 1.24 (3H, t, $J = 6.9$ Hz, CH_3 on isoxazole ring), 2.55 (3H, complex m, $CH_2 + CH$ of cyclohexene ring), 2.59 (2H, q, $J = 6.9$ Hz, CH_2 of isoxazole ring), 3.17 (1H, d, $J = 11.0$ Hz, CH on C_1), 3.67 (3H, s, OCH_3), 5.54 (1H, s, =CH), 7.40 (1H, s, CH on isoxazole ring), 8.11 (1H, bs, NH). Anal. ($C_{14}H_{18}N_2O_4$) C, H, N.

*6.4.1.5. Ethyl 4-[(3-ethyl-5-isoxazolyl)amino]-6-methyl-2-oxo-3-cyclohexene-1-carboxylate, (**18**).* In like manner to isoxazole **17**, ketone **2** and 5-amino-3-ethylisoxazole (**13**), produced **18**, m.p. 122–123 °C; yield 15% (after chromatography and three recrystallisations from 2-propanol) as white crystals. ^{1}H-NMR (DMSO d_6): δ 1.06 (3H, d, $J = 6.0$ Hz, CH_3 on C_6), 1.24 (3H, t, $J = 7.0$ Hz, CH_3 on isoxazole ring), 1.28 (3H, t, $J = 7.0$ Hz, CH_3 of ethyl group), 2.55 (3H, complex m, $CH_2 +$ CH of cyclohexene ring), 2.59 (2H, q, $J = 6.9$ Hz, CH_2 of isoxazole ring), 3.17 (1H, d, $J = 11.0$ Hz, CH on C_1), 4.20 (2H, q, $J = 7.0$ Hz, CH_2 of ethyl group), 5.54 (1H, s, =CH), 7.40 (1H, s, CH on isoxazole ring), 8.12 (1H, bs, NH). Anal. ($C_{15}H_{20}N_2O_4$) C, H, N.

*6.4.1.6. Tert-butyl 4-[(3-ethyl-5-isoxazolyl)amino]-6-methyl-2-oxo-3-cyclohexene-1-carboxylate, (**19**).* Compound **19** was prepared from ester **3** and 5-amino-3-ethylisoxazole (**13**), m.p. 102–104 °C; yield 14% (after chromatography and three recrystallisations from ethyl acetate:ether, 3:1) as light yellow crystals. ^{1}H-NMR (DMSO d_6): δ 1.06 (3H, d, $J = 6.0$ Hz, CH_3 on C_6), 1.24 (3H, t, $J = 6.9$ Hz, CH_3 on isoxazole ring), 1.40 (9H, s, $3 \times CH_3$), 2.55 (3H, complex m, $CH_2 + CH$ of cyclohexene ring), 2.59 (2H, q, $J = 6.9$ Hz, CH_2 of isoxazole ring), 3.17 (1H, d, $J = 11.0$ Hz, CH on C_1), 5.54 (1H, s, =CH), 7.40 (1H, s, CH on isoxazole ring), 8.1 (1H, bs, NH). Anal. ($C_{17}H_{24}N_2O_4$) C, H, N.

6.5. X-ray crystal analysis

Ethyl 4-[(5-methyl-3-isoxazolyl)amino]-6-methyl-2-oxo-3-cyclohexene-1-carboxylate (**10**), and methyl 4-[(5-chloro-2-amino)pyridyl]-6-methyl-2-oxo-3-cyclohexene-1-carboxylate (**21**), were recrystallised from ethanol. All experimental details related to the structural analysis are provided Tables 3 and 4. The structures were solved by direct methods of the SHELXTLPC program and refined by the SHELXTL program [33]. There was no intensity decay in the analysis.

6.6. Pharmacology

Initial evaluations for anticonvulsant activity were performed by the ADD program, Epilepsy Branch, Neurological Disorders Program, NINDS and included Phases I, II, VIA and VIB test procedures which have been described [17,18]. These tests were performed either in male Carworth Farms no. 1 (CF1) mice (weighing 18–25 g) or male Sprague–Dawley rats (weighing 100–150 g). Phase I, a qualitative anticonvulsant ip evaluation in mice included three tests: MES, scPTZ, and the rotorod test for neurological toxicity (Tox). Compounds were suspended in 0.5% aqueous methylcellulose and were administered at three dosage levels (30, 100, and 300 mg kg^{-1}) with anticonvulsant activity and motor impairment noted 30 min and 4 h after administration. Phase VIA evaluation was a similar qualitative evaluation to the Phase I evaluation, however, the test drug was administered orally in rats utilising the three tests noted previously. The Phase II test quantitated the anticonvulsant activity and motor impairment observed for the most promising compounds in Phase I and VIA, respectively. An ip test in rats was performed at 30 mg kg^{-1} for activity and toxicity. The time of peak effect ip in mice and rats was also noted for the determination of ED_{50} and TD_{50} values. All test data are listed in Table 1. Phase V of the ADD testing protocol measured the ability of compounds to provide protection against seizures induced by subcutaneous injection of the CD_{97} of bicuculline (2.7 mg kg^{-1}) and picrotoxin (3.15 mg kg^{-1}). A special ip toxicity evaluation was performed on compound **11** in Sprague–Dawley rats. The TTE test [19] is described as follows. Twenty mice were pretreated with 100 mg kg^{-1} of the test compound. At several time intervals (0.25, 0.5, 1, 2 and 4 h) post-treatment with the test compound, four mice at each time point were challenged with 12.5 mA of electrical current for 0.2 s via corneal electrodes. This stimulation produced a TTE seizure in the animals. For each time interval, results were expressed as a ratio of the number of animals protected over the number of animals tested. The procedure for hippocampal slices is as follows. A bipolar stimulating electrode was stereotactically implanted in the ventral hippocampus (AP -3.6, ML 4.9, DV -5.0 from dura, incisor bar $+5$) of adult male Sprague–Dawley rats (250–300 g) under ketamine–xylazine anesthesia. Three anchor screws were attached to the

skull and the electrode assembly anchored to the skull with dental acrylic cement. After the incision is closed with sutures, the animal received a single dose of bicillin (60 000 U, im) and returned to the home cage to recover. Animals were kindled according to the procedure of Lothman and Williamson [34]. Briefly, after 1 week, animals were stimulated with suprathreshold trains of 200 μA for 10 s, 50 Hz, every 30 min for 6 h on alternate days until the animals are fully kindled. One week later the effect of a single dose of test substance (50 mg kg^{-1}, ip) on the behavioural seizure score (BSS) and after-discharge was assessed in a single group of kindled rats ($n = 6–8$) at 15, 45, 75, 105, 135, 165, and 195 min after drug administration. Results obtained at the various time points were compared with the last control stimulus delivered 15 min prior to drug administration. Thus, each animal served as its own control. Seizures are scored according to the following criteria: Stage 1, mouth and facial clonus; Stage 2, stage 1 plus head nodding; Stage 3, stage 2 plus forelimb clonus; Stage 4, stage 3 plus rearing; Stage 5, stage 4 plus repeated rearing and falling [35]. When a drug treatment is observed to significantly lower seizure score and decrease afterdischarge, a dose–response study will be initiated. Compound **11** was studied, but did not significantly score well to qualify, however.

6.7. Sodium channel binding

The sodium channel binding assay on isoxazole compound **10** was performed by NovaScreen, Hanover, MD USA. [^{3}H]Batrachotoxinin A 20α-benzoate was prepared as described and assayed to contain a final ligand concentration of 2.0 nM [20]. The sodium channel binding assay was similar to reported methods [20]. Reactions were carried out in 50 nM HEPES (pH 7.4 at 25 °C) containing 130 nM choline chloride at 37 °C for 45 min. The reaction was terminated by rapid vacuum filtration of the reaction mixture onto glass fibre filters. Radioactivity trapped onto the filters was determined and compared with control values in order to ascertain any interaction of test compounds with the sodium site 2 binding site. The IC_{50} value (concentration of compound required to inhibit 50% of specific neurotoxin binding) were determined from a dose–response curve generated by plotting the log of anticonvulsant concentration (over a range of 10–800 mM) versus percent of specifically bound [^{3}H]BTX-B. Isoxazole compound **10** was inactive in this test.

6.8. Pharmacokinetics

6.8.1. BBMECs—isolation and seeding

BBMEC were isolated manually from the gray matter of cerebral cortices as described by Audus and Borchardt [27,36]. Approximately two to three bovine brains were obtained and the minced gray matter of the cortex was collected and placed in a sterile vessel, suspended to a final volume of 500 mL in serum free minimum essential medium (MEM, pH 9.0) containing dispase. The final concentration of the suspension was 0.5% (w/v). This suspension was placed in a shaking water bath (100 oscillations per min) for 3 h at 37 °C. The crude capillary pellets remaining after two successive centrifugations was resuspended in 20 mL of MEM containing 1 mg mL^{-1} of collagenase/dispase and placed on a shaking water bath for 3 h at 37 °C. The capillary suspension was diluted to 50 mL in MEM and centrifuged. The pellet was resuspended in 8–10 mL of MEM. Aliquots (2–3 mL) of the suspension were layered over 45 mL of a 50% Percol gradient in each of four 50-mL centrifuge tubes and centrifuged for 10 min at 4 °C. The second band was removed via pipette, placed into four separate centrifuge tubes, and diluted in MEM up to 50 mL. Final capillary pellets were obtained by centrifugation for 10 min. The primary bovine microvessel fragments were then seeded onto 12 well Costar inserts (Transwell). Filter membranes (AP surface) of inserts were treated with rat tail collagen type 1 (ca. Two to three drops), and allowed to incubate for 30 min in NH_3 fumes. Fibronectin (0.5 mL) was then added to each insert, removed after 40 min and allowed to dry. BBMECs were then seeded onto the collagen/fibronectin-coated surfaces at a density of ca. 25×10^6 cells per cm^2. Upper (AP) compartments received 0.5 mL of plating medium and the lower (BL) received 1.5 mL for the first 3 days of growth and every other day thereafter. Cells were placed under standard culture conditions (37 °C, 5% CO_2), and 95% humidity until confluence was reached within 14–15 days.

6.8.2. Monolayer integrity

Monolayer integrity of both BBMECs was assessed by noting the flux of radiolabelled markers [^{14}C]sucrose (paracellular) and [^{3}H]propranolol (transcellular) for each 12 well Costar plate before the transport experiments.

6.8.3. Transport studies

Enaminone solutions were prepared in DMSO ($<$ 2%) and PBS reaching a final concentration of ca. 1×10^{-4} M. Upon confluency of both BBMECs, complete culture medium was removed from both the AP and BL sides and washed twice with sterile PBS. Transwell inserts were placed into tissue culture plates containing 1.5 mL of prewarmed PBS for permeability studies. The appropriate enaminone solution [isoxazole **10** or prototype **20a** ($R^3 = 4$-Cl)] radiolabelled marker, or blank buffer was added to either the AP or BL side. All transport experiments were performed at 37 °C in PBS. BBMECs were continuously agitated on a plate during transport experiments (100 cycles per min). For

examination of transport in the AP → BL direction, the Transwell inserts were moved into wells with fresh PBS. At time ($t = 0$), 400 μL of drug was added to the AP side of the monolayer. For examination in the BL → AP direction, drug (1500 μL) was initially added to the donor side at time ($t = 0$) and at times between 5 and 120 min. Complete amounts of sample from the AP side were removed and replaced with fresh prewarmed PBS.

6.8.4. Inhibition studies

Inhibition studies using either verapamil (50 μM), probenecid (100 μM) or indomethacin (10 μM) were performed to examine the possible interaction of the enaminones with possible efflux mechanisms on the BBMEC monolayers (i.e. Pgp and MDR). Transport studies were performed as previously indicated. After removal of the medium and washing with PBS, Transwell inserts were placed in fresh buffer and preincubated with inhibitor on both the AP or BL side at 37 °C for 30–45 min. Drug was then added to the donor side at time ($t = 0$), and Transwell inserts were removed after 120 min. All samples were removed after 120 min and kept frozen (-70 °C) until HPLC analysis.

6.8.5. HPLC analysis

Reverse phase chromatography with ultraviolet detection ($\lambda = 307$ nm) was used to quantitate the eluate. The chromatographic system: Model 515 liquid chromatograph (Waters-Millipore, Milford, MA, USA); 717 Waters autosampler; Waters model 486 UV detector and a 3390A Hewlett Packard integrator (Avondale, PA, USA). Enaminone standards ranged from 0.1 to 50 μg mL^{-1} and the internal standard used was carbamazepine (5 μg mL^{-1}). The analytical column was an ODS C18 (250 × 4.6 mm, Phenomenex, Torrance, CA, USA). The buffer component of the mobile phase (0.05 M phosphate buffer) was prepared with deionised water and the pH was adjusted to 7.0. the mobile phase was filtered through a 0.45 μM nylon filter and degassed under ultrasound and vacuum for 15 min. The mobile phase consisted of acetonitrile:0.05 M phosphate buffer (65:35, v/v). The assay was linear in concentration ranges of 0.1 to 50 μg mL^{-1} ($r > 0.999$) and interday precision ranged from 0.5 to 5.5%.

6.8.6. Data analysis

The apparent permeability coefficients (P_{app}) of the enaminones alone or in the presence of inhibitors were determined following triplicate experiments. Permeability coefficients were determined at sink conditions from the following equation:

$$P_{app} = \frac{\Delta Q}{\Delta t}\frac{1}{AC_{(0)}}$$

where $\Delta Q/\Delta t$ is equal to the linear appearance rate of mass in the receiver solution; A, the cross sectional area and $C_{(0)}$ is the initial enaminone concentration in the donor compartment [37]. All values are represented as mean and standard deviation of the values from three monolayer Transwell inserts prepared under identical conditions and from the same preparation of cells. The results of the evaluations of isoxazole **10** and the anilino prototype, **20a** (R^3 = 4-Cl) are shown on Table 5.

7. Supplementary material

Crystal structures have been deposited at the Cambridge Crystallographic Data Centre and allocated the deposition number CCDC number 182109 and 182110.

References

[1] F. Lepage, F. Tombret, G. Cuvier, A. Marivain, J.M. Gillardin, Eur. J. Med. Chem. 27 (1992) 581–593.
[2] J.P. Stables, H.J. Kupferberg, The NIH anticonvulsant drug development (ADD) program: preclinical anticonvulsant screening project, in: G. Avanzini, P. Tanganelli, M. Avoli (Eds.), Molecular and Cellular Targets for Anti-Epileptic Drugs, John Libbey and Co, 1997, pp. 191–198.
[3] I.O. Edafiogho, C.N. Hinko, H. Chang, J.A. Moore, D. Mulzac, J.M. Nicholson, K.R. Scott, J. Med. Chem. 35 (1992) 2798–2805.
[4] K.R. Scott, I.O. Edafiogho, E.L. Richardson, V.A. Farrar, J.A. Moore, E.I. Tietz, C.N. Hinko, E. Chang, A. El-Assadi, J.M. Nicholson, J. Med. Chem. 36 (1993) 1947–1955.
[5] K.R. Scott, G.O. Rankin, J.M. Stables, M.S. Alexander, I.O. Edafiogho, V.A. Farrar, K.R. Kolen, J.A. Moore, L.D. Sims, A.D. Tonnu, J. Med. Chem. 38 (1995) 4033–4043.
[6] M.L. Laws, R.R. Roberts, J.M. Nicholson, R. Butcher, J.P. Stables, A.M. Goodwin, C. Smith, K.R. Scott, Bioorg. Med. Chem. 6 (1998) 2289–2299.
[7] J.E. Foster, J.M. Nicholson, R. Butcher, J.P. Stables, I.O. Edafiogho, A.M. Goodwin, M.C. Henson, C.A. Smith, K.R. Scott, Bioorg. Med. Chem. 7 (1999) 2415–2425.
[8] R.R. Roberts, I.O. Edafiogho, K.R. Scott, US Patent No. 5,580,894 (1996).
[9] T. Tatee, K. Narita, S. Kurashige, S. Ito, H. Miyazaki, H. Yamanaka, M. Mizugaki, T. Sakamoto, H. Fukuda, Chem. Pharm. Bull. 34 (1986) 1643–1655.
[10] O. Foussard-Blanpin, M. Ray, Ann. Pharmaceutiques Francaises 40 (1982) 339–350.
[11] I.O. Edafiogho, K.R. Scott, Anticonvulsants, in: M.E. Wolff (Ed.), Burger's Medicinal Chemistry and Drug Discovery, fifth ed., Wiley, New York, 1996, pp. 175–260.
[12] T. Nishiwaki, T. Saito, J. Chem. Soc. (C) (1971) 3021–3026.
[13] R.J. Friary, J.M. Gilligan, R.P. Szajewski, K.J. Falci, R.W. Franck, J. Org. Chem. 38 (1973) 3487–3491.
[14] I.O. Edafiogho, J.A. Moore, M.S. Alexander, K.R. Scott, J. Pharm. Sci. 83 (1994) 1155–1170.
[15] I.O. Edafiogho, Personal communication.
[16] F.H. Allen, O. Kennard, Chem. Design Automation News 8 (1993) 31–38.
[17] R.L. Krall, J.K. Penry, B.G. White, H.J. Kupferberg, E.A. Swinyard, Epilepsia 19 (1978) 409–428.

[18] R.J. Porter, J.J. Cereghino, G.D. Gladding, B.J. Hessie, H.J. Kupferberg, B. Scoville, B.G. White, Cleveland Clin. Q. 51 (1984) 293–305.
[19] S.G. Piredda, J.H. Woodhead, E.A. Swinyard, J. Pharmacol. Exp. Ther. 232 (1985) 741–745.
[20] C.R. Creveling, Mol. Pharmacol. 23 (1983) 350–358.
[21] V.L. Trainer, E. Moreau, D. Guedin, D.G. Baden, W.A. Catterall, J. Biol. Chem. 268 (1993) 17114–17119.
[22] N.D. Eddington, D.S. Cox, R.R. Roberts, J.P. Stables, C.B. Powell, K.R. Scott, Curr. Med. Chem. 7 (2000) 417–436.
[23] D.S. Cox, K.R. Scott, H. Gao, S. Raje, N.D. Eddington, J. Pharm. Sci. 90 (2001) 1540–1555.
[24] M.V. Shah, K.L. Audus, R.T. Borchardt, Pharm. Res. 6 (1989) 624–627.
[25] W.M. Pardridge, Adv. Drug Del. Rev. 15 (1995) 5–36.
[26] E.P. Eddy, B.E. Maleef, T.K. Hart, P.L. Smith, Adv. Drug Del. Rev. 23 (1997) 185–198.
[27] K.L. Audus, R.T. Borchardt, Ann. NY Acad. Sci. 507 (1987) 9–18.
[28] MACLOGP Program; version 4.0, BioByte Corp. Claremont, CA 1711 USA.
[29] A.P. Oliver, B.J. Hoffer, R.J. Wyatt, Epilepsia 18 (1977) 543–548.
[30] S. Piredda, W.D. Yonekawa, T.S. Whittingham, J.J. Kupferberg, Epilepsia 26 (1985) 167–174.
[31] H.J. Kupferberg, J.P. Stables, Mechanisms of action revisited: drug discovery, testing and clinical prediction, in: H. Stefan, G. Kramer, B. Mamoli (Eds.), Challenge in Epilepsy—New Anticonvulsant Drugs, Blackwell Science Ltd, 1998, pp. 7–27.
[32] C. Hansch, J.P. Bjorkroth, A. Leo, J. Pharm. Sci. 76 (1987) 663–687.
[33] G.M. Sheldrick, SHELXLTLPC and SHELXTL, Program for crystal structure determination, Cambridge University, England, 1996.
[34] E.W. Lothman, J.M. Williamson, Brain Res. 649 (1994) 71–81.
[35] R.J. Racine, Electroenceph. Clin. Neurophysiol. 32 (1972) 281–294.
[36] K.L. Audus, R.T. Borchardt, Pharm. Res. 3 (1986) 81–87.
[37] P. Couraud, D. Scherman, Biology and Physiology of the Blood–Brain Barrier. Transport, Cellular Interaction, and Brain Pathologies, Plenum Press, New York (1996).