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Synthesis, acetylcholinesterase inhibition and neuroprotective activity of new tacrine analogues

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Abstract—The synthesis and the biological evaluation (acetylcholinesterase inhibition activity and neuroprotection) of the new tacrine analogues **2**–**14** is described.

1. Introduction

Alzheimer's disease (AD) is the most common type of dementia in western societies.[1] Of the many pathogenic hypotheses of AD (i.e., β-amyloid deposits, hyperphosphorylation of protein τ, cholinergic neuron loss in the basal nucleus of Meynert[2]) an alteration of cell Ca^{2+} homeostasis might be at the centre of the metabolic crossroad leading neurons to apoptotic death.[3,4] The neuropsychiatric symptoms associated with the illness are related to the reduction in the number of functional neuronal nicotinic receptors (nAChR);[5] for this reason the only therapeutical strategy that, up to now, has proven to have some efficacy in slowing progression of AD is that which improves cholinergic neurotransmission, counteracting the deficit of cerebral acetylcholine.[6] Therefore, acetylcholinesterase (AChE) inhibition is currently the therapeutic strategy most commonly used to treat Alzheimer patients. On the other hand, the multifactorial pathogenesis of AD suggests that drug treatments with two or more mechanisms of action, acting in a complementary manner, could be more efficacious to patients suffering the disease. In fact, galanthamine, a drug currently used to treat AD, is a mild inhibitor of AChE that sensitizes the nicotinic receptor to AChE[7] and prevents cell death induced by β-amyloid or thapsigargin.[8] On the other hand, tacrine (**1**, Chart 1), a potent and reversible AChE inhibitor, was the first drug approved in the United States for the palliative treatment of AD, but the poor selectivity of this drug for AChE resulted in a number of side effects, specially hepatotoxicity,[6b] and current research is focused on developing new AChE inhibitors with improved activity and reduced adverse side effects.

In this context, a few years ago, we started a project aimed at the synthesis and the pharmacological study of new tacrine analogues. As a result, we obtained a number of compounds of class **A** and **B**, with a substituted benzene-moiety at C-4 position, that keep their AChE inhibitory properties and behave as mild Ca^{2+} promotors, enhancing the entry of Ca^{2+} into excitable (chromaffin) and smooth muscle cells (Chart 1).[9]

Continuing with this project we now report the synthesis of compounds **2**–**14** with a 3- or 4-pyridyl, 2-(*N*-acetyl)-pyrrolyl and 2-thienyl groups at position C-4 (Chart 1). In these tacrine analogues we wanted to evaluate the biological activity of related substrates with a heterocyclic ring moiety at the C4-position. We also describe some aspects of their biological profile, like the acetylcholinesterase (AChE) inhibition activity and their properties as neuroprotector agents.

2. Results and discussion

2.1. Chemistry

The synthesis of these new tacrine analogues **2**–**11** and **12**–**14** has been achieved by Friedländer reaction[10] of

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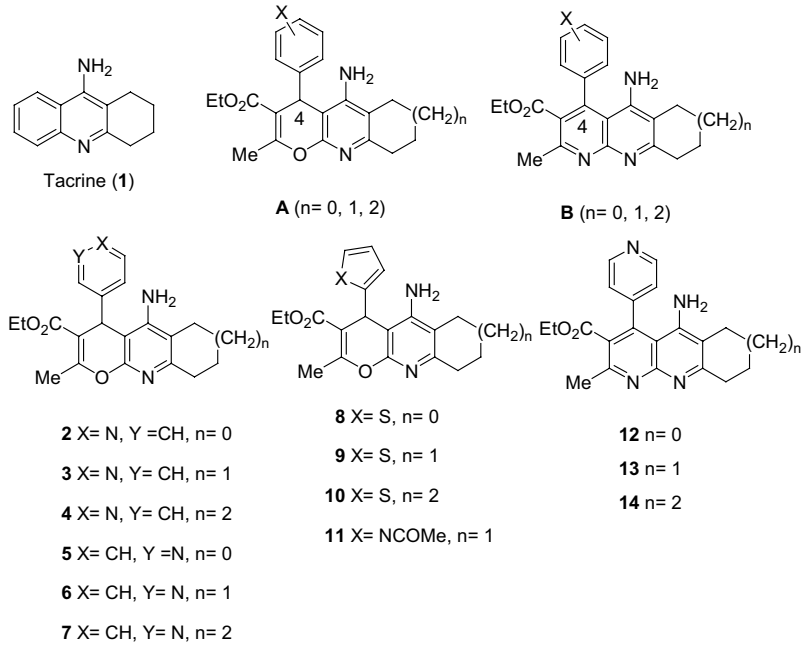


Chart 1.

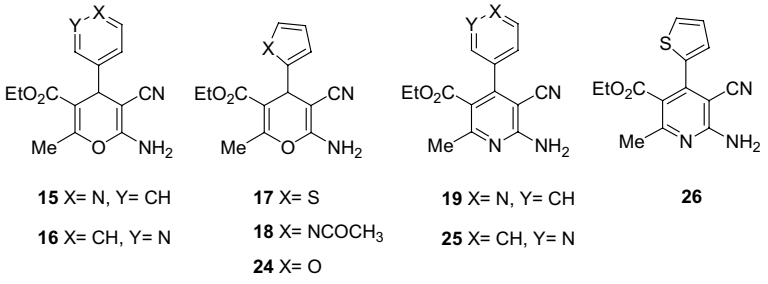


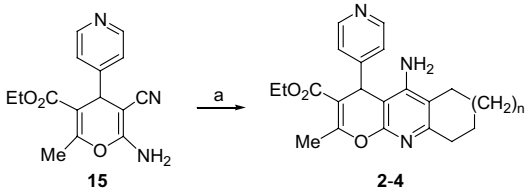
Chart 2.

the corresponding heterocyclic precursors, such as, ethyl 6-amino-5-cyano-4*H*-pyran-3-carboxylates[11] (**15**–**18**) (Chart 2) and ethyl 6-amino-5-cyano-4-pyridine-3-carboxylate[12] (**19**), respectively (Chart 2) with selected ketones (cyclopentanone, cyclohexanone or cycloheptanone).

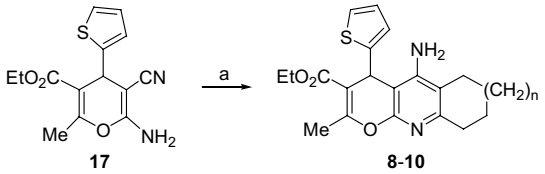
For the pyran-like tacrine derivatives **2**–**11** (Chart 1), under the standard conditions,[10] using dry 1,2-dichloroethane as solvent and aluminium trichloride as promotor, known ethyl 6-amino-5-cyano-4-(4-pyridyl)-4*H*-pyran-3-carboxylate **15**[13] (Chart 2) and ethyl 6-amino-5-cyano-4-(2-thienyl)-4*H*-pyran-3-carboxylate **17**,[13] (Chart 2) afforded the expected tacrine analogues **2**–**4** (Scheme 1) and **8**–**10** (Scheme 2), respectively, after reaction with cyclopentanone, cyclohexanone and cycloheptanone, in mild conditions and moderate chemical yields.

Pyran **16** (Chart 2) is available from commercial sources in small amounts (Akos, Lithuania), but very surprisingly it has never been reported in the literature. We have prepared it using the standard protocol[12] by 'one-pot' reaction from commercially available 3-pyridinecarboxaldehyde (**20**), via the Knoevenagel condensation product 3-pyridylmethylidenemalononitrile (**21**),[14] not isolated, after in situ Michael addition with ethyl acetoacetate, catalyzed by piperidine (Scheme 3). Finally, the Friedländer reaction[10] of compound **16** with cyclopentanone, cyclohexanone and cycloheptanone gave products **5** (73%), **6** (88%) and **7** (77%), respectively (Scheme 3).

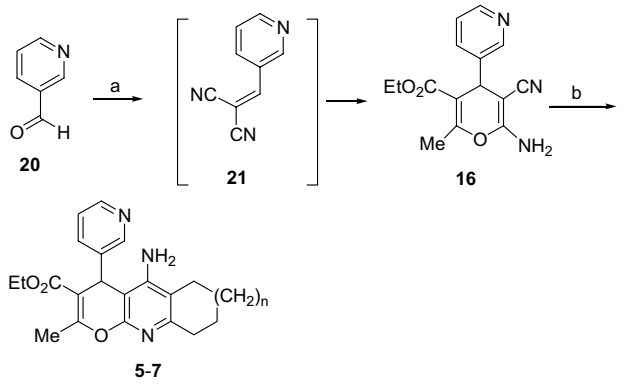
Similarly, pyran **18** (Chart 2) has been prepared for the first time by Michael reaction of ethyl acetoacetate and *N*-acetyl-2-pyrrolylmethylidenemalononitrile (**23**), that we were able to synthesize by simple acetylation of the



Scheme 1. Reagents and conditions: (a) 1,2-dichloroethane, $AlCl_3$, cyclopentanone (**2**, $n = 0$, 65%); cyclohexanone (**3**, $n = 1$, 85%), cycloheptanone (**3**, $n = 2$, 97%).



Scheme 2. Reagents and conditions: (a) 1,2-dichloroethane, $AlCl_3$, cyclopentanone (**8**, $n = 0$, 29%); cyclohexanone (**9**, $n = 1$, 83%), cycloheptanone (**10**, $n = 2$, 61%).



Scheme 3. Reagents and conditions: (a) malononitrile, then ethyl acetoacetate, piperidine (84%); (b) 1,2-dichloroethane, $AlCl_3$, cyclopentanone (**5**, $n = 0$, 73%); cyclohexanone (**6**, $n = 1$, 88%), cycloheptanone (**7**, $n = 2$, 77%).

previously described 2-pyrrolylmethylidenemalononitrile (**22**)[15] (Scheme 4). Finally, the Friedländer reaction of compound **18** with cyclohexanone gave product **11** (43%) (Scheme 4). The reaction of pyran **18** (Chart 2) with cyclopentanone or cycloheptanone failed.

We have also prepared ethyl 6-amino-5-cyano-4-(2-furyl)-2-methyl-4*H*-pyran-3-carboxylate (**24**)[10a,b,16] (Chart 2), but we were unable to perform any successful Friedländer reaction on this substrate, probably due to the lability of the furan derivatives under these experimental conditions.

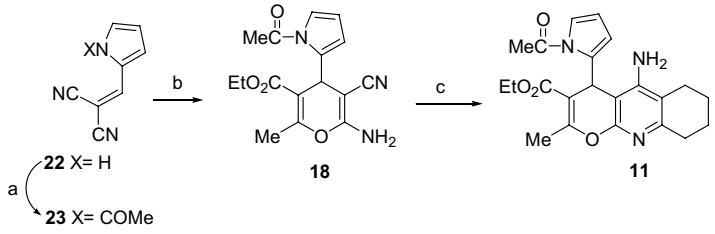
The naphthyridine-like tacrine derivatives **12**–**14** (Chart 1) have been synthesized from pyridine **19**, prepared from the corresponding pyran **15**[13] as usual, after reaction with ammonium acetate in acetic acid,[12] as shown in Scheme 5.

The new pyridines **25** and **26** (Chart 2), prepared from the corresponding 4*H*-pyrans **16** (Scheme 5) and **17** (Scheme 6), respectively,[12] did not afford the expected tacrine analogues.

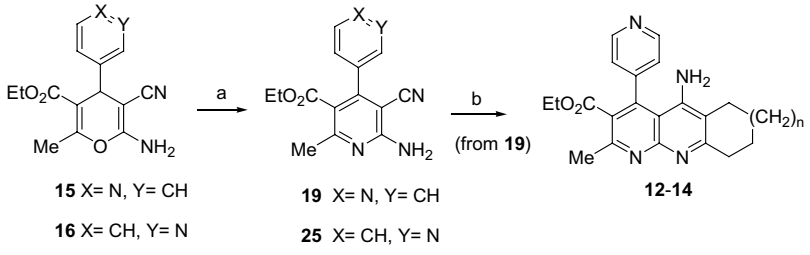
2.2. Pharmacology

2.2.1. The AChE inhibitory activity. According to the standard methodology[17] we obtained the following data for the acetylcholinesterase inhibition activity in the pyrano[3,2-*e*]pyridines, and pyrano[2,3-*b*]quinolines **2**–**10** (see Table 1). For comparative purposes we have also included in Table 1 the IC_{50} (μM) for tacrine (**1**) and the previously published analogues **27** and **28**[9b] (Chart 3). None of the test compounds showed an improved AChE inhibition as compared to tacrine (IC_{50} 0.14 mM), with compound **27** (IC_{50} 0.87 mM)[9b] being the most active in this assay.

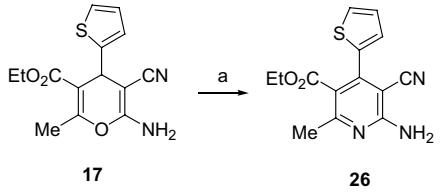
Structure–activity relationship analysis regarding the heterocyclic ring moiety in the C4-position of tacrine leads to the following conclusions. For the class **A** (i) the pyrrolyl containing substrate (**11**) is the most active (10-fold drop compared to tacrine), followed by the 4-pyridyl containing substrates (**2**–**4**). The 3-pyridyl—with the exception of compound **6**—and 2-thienyl derivatives



Scheme 4. Reagents and conditions: (a) Ac_2O, py (98%); (b) ethyl acetoacetate, piperidine (99%); (c) 1,2-dichloroethane, $AlCl_3$, cyclohexanone (43%).



Scheme 5. Reagents and conditions: (a) AcOH, $AcONH_4$ (**15**–**19**: 57%), (**16**–**25**: 15%); (b) 1,2-dichloroethane, $AlCl_3$, cyclopentanone (**12**, $n = 0$, 61%); cyclohexanone (**13**, $n = 1$, 49%), cycloheptanone (**14**, $n = 2$, 92%).



Scheme 6. Reagents and conditions: (a) AcOH, $AcONH_4$ (5%).

Table 1. IC_{50} (μM) values for the AChE inhibition

	n	Heterocycle	IC_{50} (μM)
Tacrine (**1**)		—	0.14
2	0	4-Pyridyl	6.6
5	0	3-Pyridyl	>100
8	0	2-Thienyl	27.8
27[9b]	1	4-(OMe)C_6H_4	0.87
3	1	4-Pyridyl	3.0
6	1	3-Pyridyl	7.11
9	1	2-Thienyl	24.8
11	1	2-Pyrrolyl	1.7
4	2	4-Pyridyl	8.6
7	2	3-Pyridyl	32.3
10	2	2-Thienyl	14.4
28[9b]	1	C_6H_5	0.82
12	0	4-Pyridyl	8.2
13	1	4-Pyridyl	1.4
14	2	4-Pyridyl	5.1

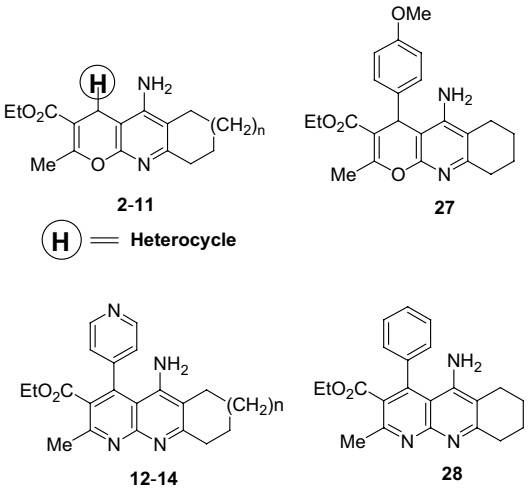


Chart 3.

are poor AChE inhibitors (IC_{50} > 10 mM). (ii) The six-membered cycloannulated compounds (*n* = 1) (**3**, **6**, **9**) were more potent than their five- and seven-membered analogues. In those cases where it was possible to prepare the three cycloalkyl fused-ring onto the central 4-aminopyridine nucleus (*n* = 0, *n* = 1, *n* = 2), the most active compounds were always the 4-pyridyl derivatives (**2**–**4**). This was particularly obvious for compounds **2** (4-pyridine substituent; IC_{50} 6.6 mM) and its close analogue compound **5** (3-pyridine substituent; IC_{50} > 100 mM).

Regarding the [1,8]naphthyridine derivatives **12**–**14** (class **B**), it can be seen that the replacement of a benzene substituent, compound **28** (Chart 3),[9b] for a 4-pyridyl substituent is not favoured. Again, the six-membered cycloannulated compound shows the strongest inhibitory effect. This compound is in fact the most potent in the whole series synthesized. Comparing the two classes, the [1,8]naphthyridine derivatives (class **B**) are the more potent one.

2.2.2. Neuroprotection. Inhibition of AChE may temporarily improve AD symptoms such as memory and cognitive impairment,[18] but not the ongoing cellular loss. Drug treatments with two or more mechanisms of action, acting in a complementary manner, could be more efficacious to patients suffering from the disease. Galanthamine, which is an allosteric potentiating ligand of the nAChR,[7] also prevents apoptotic cell death induced by different toxic stimuli.[8] We decided to evaluate the potential cytoprotective effect of the new compounds and several intermediates on bovine chromaffin cells, a type of cells that possess all the characteristics and types of Ca^{2+} channels of sympathetic neurons, exposed during 24 h to veratridine 30 μM, a toxin, which induces a Ca^{2+} overload and a consequent cell death.[19]

Drugs, at the concentration of 3 μM, were administered 24 h before the incubation of cells with veratridine and maintained during the whole of the experiment. After that, release of lactic dehydrogenase (LDH) was measured as a parameter of cell death, since this enzyme is released to the extracellular medium as a consequent cell death.[19] Basal release of LDH by the cells was subtracted from the values obtained for all the compounds or veratridine. Results are shown in Table 2.

Only six of the new compounds showed a tendency of some neuroprotective action, which-at the drug concentration tested-was statistically significant only in the case of compound **10** and intermediate **16**. None of the test compounds was as effective as the previously synthesized compound **28**,[9b] shown here for comparison.

3. Conclusions

To summarize, we have reported the synthesis, acetylcholinesterase (AChE) inhibitory activities and possible neuroprotective properties of new tacrine analogues (**2**–**14**). With respect to inhibition of AChE, none of the test compounds showed superior activity to tacrine (IC_{50} 0.14 mM). For the pyrano[3,2-*e*]pyridines, and pyrano[2,3-*b*]quinolines group (class **A**), test compound (**11**) (IC_{50} 1.7 mM) and for the [1,8]naphthyridine derivatives, compound **13** (IC_{50} 1.4 mM) were the most potent. In addition, comparing our current data with the ones obtained in previous works from these laboratories,[9] it is clear that the incorporation of heterocyclic rings systems on these substrates, still gives us compounds with some AChE inhibitory profile, in the μM range, but significantly less active compared with the simple benzene-substituted derivatives.[9] Regarding neuroprotective properties of the different tacrine ana-

Table 2. Cell viability expressed as increase of LDH released in the presence of veratridine

Compound (3 μM)	LDHf (% total)	% Protection
Vehicle	31.2 ± 1.1	
Tacrine	30.8 ± 2.9	1.3
2	35.1 ± 2.9	0
3	30.6 ± 4.7	1.9
4	30 ± 4	3.8
5	32.2 ± 4.4	0
6	32.5 ± 3.2	0
7	**24** ± 4.5	**23.1**
8	30.4 ± 2.1	2.6
9	**28.4** ± 1.7	**9**
10	**18.7** ± 4	**40.1*****
11	**28** ± 1.8	**10.3**
12	31.6 ± 1.8	0
13	30.7 ± 0.8	1.6
14	**28.2** ± 1	**9.6**
15	31.9 ± 2.1	0
16	**19.8** ± 1.4	**36.5*****
17	29.8 ± 2.1	4.5
18	30.3 ± 2.5	2.9
19	30.7 ± 2	1.6
25	30.2 ± 1.7	3.2
26	29.7 ± 1.4	4.8
28[9b]	**17.4** ± 1.4	**44.2*****

*** $p \leqslant 0.001$.

logues, some showed a clear tendency in reducing the veratridine-induced LDH release at the concentration tested. This effect was statistically significant only for compound **10** and intermediate **16**. Unfortunately, none of the new tacrine analogues showed a profile in which these two pharmacological parameters were combined.

4. Experimental part

4.1. General methods

Reactions were monitored by TLC using precoated silica gel aluminium plates containing a fluorescent indicator (Merck, 5539). Detection was done by UV (254 nm) followed by charring with sulfuric–acetic acid spray, 1% aqueous potassium permanganate solution or 0.5% phosphomolybdic acid in 95% EtOH. Anhydrous Na_2SO_4 was used to dry organic solutions during work-ups and the removal of solvents was carried out under vacuum with a rotary evaporator. Flash column chromatography was performed using silica gel 60 (230–400 mesh, Merck). Melting points were determined on a Kofler block and are uncorrected. IR spectra were obtained on a Perkin–Elmer Spectrum One spectrophotometer. ^{1}H NMR spectra were recorded with a Varian VXR-200S spectrometer, using tetramethylsilane as internal standard and ^{13}C NMR spectra were recorded with a Bruker WP-200-SY. All the assignments for protons and carbons were in agreement with 2D COSY, gHSQC, gHMBC and 1D NOESY spectra. Elemental analyses were conducted on a Carlo Erba EA 1108 apparatus. All new compounds showed analytical and spectroscopic data in good agreement with these structures, and with related molecules, previously synthesized in our laboratory.[9]

4.2. General method for the synthesis of ethyl 6-amino-4-aryl-5-cyano-2-methyl-4*H*-pyran-3-carboxylates

To a solution of the corresponding aldehyde (1 equiv) in dry toluene (1 mL/mmol), under argon, malononitrile (1 equiv) and a catalytic amount of piperidine were added. The mixture was stirred at rt for 3–7 h. The solvent was evaporated and the crude was purified by column chromatography, eluting with mixtures of hexane/ethyl acetate. Next, to a solution of this arylidenemalononitrile in dry toluene, ethyl acetoacetate (1 equiv) and a catalytic amount of piperidine were added. The mixture was stirred at rt for 2 h, and the precipitated solid was isolated by filtration, washed with cold toluene, dried and recrystallized.

4.2.1. General method for the synthesis of ethyl 6-amino-4-aryl-5-cyano-2-methyl-pyridine-3-carboxylates. To a solution of ammonium acetate (10 equiv) in acetic acid (1.25 mL) the appropriate ethyl 6-amino-5-cyano-2-methyl-4*H*-pyran-3-carboxylate (1 equiv) was added. The mixture was refluxed (bath temperature at 116 °C) for 3–7 h. Then, the reaction was cooled and the solvent was evaporated. The crude was treated with an aqueous saturated solution of sodium bicarbonate until pH 7 and extracted with ethyl acetate. The organic phase was dried with sodium sulfate, filtered and the solvent was removed. The crude was purified by column chromatography eluting with mixtures of dichloromethane/ethyl acetate. The isolated product was recrystallized from mixtures of hexane/dichloromethane.

4.2.2. General method for the Friedländer reaction. Aluminium chloride (1.2–1.7 equiv) was suspended in dry 1,2-dichloroethane (10 mL) at rt under argon. The corresponding ethyl 6-amino-4-aryl-5-cyano-2-methyl-4*H*-pyran 3-carboxylate (1 equiv) and the ketone (cyclopentanone, cyclohexanone or cycloheptanone; 1.2–1.7 equiv) were added. The reaction mixture was heated under reflux (10–24 h). When the reaction was over (TLC analysis), a mixture of THF/H_2O (2:1) was added at rt. An aqueous solution of sodium hydroxide (10%) was added dropwise to the mixture until the aqueous solution was basic. After stirring for 30 min, the mixture was extracted three times with dichloromethane. The organic layer was washed with brine, dried over anhydrous sodium sulfate, filtered and the solvent was evaporated. The resultant solid was purified by silica gel flash chromatography using methanol/dichloromethane mixtures as eluent to give pure compounds.

4.3. Ethyl 6-amino-5-cyano-2-methyl-4-(3-pyridyl)-4*H*-pyran-3-carboxylate (16)

Following the General method, 3-pyridinecarboxaldehyde (1.40 g, 13.0 mmol), malononitrile (0.858 g, 13.0 mmol), ethyl acetoacetate (1.69 g, 13.0 mmol), piperidine (15 drops), ethanol (20 mL), after 3.5 h, compound **16** (3.0 g, 84%) was obtained: mp 180–183 °C; IR (KBr) ν 3342, 3041, 2196, 1724, 1684, 1614, 1221, 1067,

714 cm^{-1}; ^{1}H NMR (DMSO-d_6, 200 MHz) δ 8.04 (d, J = 4.7 Hz, 1H), 8.38 (s, 1H), 7.54 (dt, J = 4.7 Hz, J = 6.7 Hz, 1H), 7.35 (t, J = 6.7 Hz, 1H), 7.03 (s, 2H), 4.34 (s, 1H), 3.95 (q, J = 6.9 Hz, 2H), 2.32 (s, 3H), 1.00 (t, J = 6.6 Hz, 3H); ^{13}C NMR (DMSO-d_6, 50.3 MHz) δ 165.9, 159.2, 158.3, 149.3, 148.8, 141.1, 135.5, 124.5, 120.2, 106.9, 60.9, 57.1, 37.2, 19.0, 14.4; MS (ES) [M+1]$^+$ 316 [M+Na]$^+$ 338.0; [2M+Na]$^+$ 653.3. Anal. Calcd for $C_{15}H_{15}N_3O_3$: C, 63.15; H, 5.30; N, 14.73. Found: C, 63.26; H, 5.45; N, 14.83.

4.4. *N*-Acetyl-2-pyrrolylmethylidenemalononitrile (23)

To a solution of **22**[12] (200 mg, 1.40 mmol) in pyridine (1 mL), acetic anhydride (1 mL) was added. After 9 h the solvent was evaporated and the resultant oil was purified by silica gel flash chromatography using dichloromethane as eluent to give the pure compound **23** (245 mg, 98%) as an amorphous solid: IR (KBr) ν 3433, 3142, 2219, 1722, 1576, 1446, 1375, 1280, 1259, 1127, 1078, 957 cm^{-1}; ^{1}H NMR ($CDCl_3$, 200 MHz) δ 8.79 (s, 1H), 7.78 (d, J = 3.7 Hz, 1H), 7.49 (d, J = 3.7 Hz, 1H), 6.54 (t, J = 3.7 Hz, 1H), 2.68 (s, 3H); ^{13}C NMR ($CDCl_3$, 50.3 MHz) δ 169.9, 148.4, 128.7, 128.7, 124.3, 114.9, 114.6, 114.0, 78.5, 24.7; EM (ES): [M+1]$^+$ 186, [M+Na]$^+$ 208, [2M+Na$^+$] 393. Anal. Calcd for $C_{10}H_7N_3O$: C, 64.86; H, 3.81; N, 22.69. Found: C, 64.63; H, 4.01; N, 22.58.

4.5. Ethyl 4-(1′-acetyl-1′*H*-pyrrol-2-yl)-6-amino-5-cyano-2-methyl-4*H*-pyran-3-carboxylate (18)

Following the General method, compound **23** (300 mg, 1.62 mmol) was treated with ethyl acetoacetate (211 mg, 1.62 mmol), triethylamine (18 drops), in dry toluene (3 mL) for 72 h, to give compound **18** (510 mg, 99%) as an amorphous solid: IR (KBr) ν 3408, 3311, 3197, 2192, 1717, 1671, 1637, 1605, 1384, 1369, 1305, 1120 cm^{-1}; ^{1}H NMR (DMSO-d_6, 200 MHz) δ 7.30 (d, J = 3.3 Hz, 1H), 6.75 (br s, 2H), 6.14 (dd, J = 3.2 Hz, J = 3.3 Hz, 1H), 5.94 (d, J = 3.2 Hz, 1H), 5.48 (s, 1H), 3.89 (q, J = 6.7 Hz, 2H), 2.56 (s, 3H), 2.28 (s, 3H), 0.98 (t, 3H); ^{13}C NMR (DMSO-d_6, 50.3 MHz) δ 170.5, 166.2, 159.4, 157.0, 140.4, 122.6, 120.1, 113.1, 111.5, 107.2, 60.5, 57.4, 30.5, 24.9, 18.5, 14.3; MS (APCI+) m/z [M+1]$^+$ 316, [M+Na]$^+$ 338.0, [2M+Na]$^+$ 653.3. Anal. Calcd for $C_{16}H_{17}N_3O_4$: C, 60.94; H, 5.43; N, 13.33. Found: C, 61.06; H, 5.70; N, 13.65.

4.6. Ethyl 6-amino-5-cyano-2-methyl-4-(4-pyridyl)-3-pyridinecarboxylate (19)

Following the General method, from compound **15** (1.00 g, 3.5 mmol), ammonium acetate (2.70 g, 33.06 mmol), acetic acid (44 mL), after 21 h, product **19** (203.9 mg, 57%) was obtained: mp 229–231 °C; IR (KBr) ν 3390, 3320, 3175, 2221, 1711, 1651, 1554, 1377, 1282, 1189 cm^{-1}; ^{1}H NMR (DMSO-d_6, 200 MHz) δ 8.80 (d, J = 5.5 Hz, 2H), 7.60 (s, 2H), 7.46 (d, J = 5.4 Hz, 2H), 3.98 (q, J = 7.0 Hz, 2H), 2.54 (s, 3H), 0.86 (t, 3H); ^{13}C NMR (DMSO-d_6, 50.3 MHz) δ 164.8, 159.8, 158.4, 150.4, 148.5 (2C), 143.1, 121.3 (2 × C), 114.9, 114.1, 85.4, 59.5, 22.3, 11.8; MS (APCI+) m/z [M+1]$^+$ 283, [M+Na$^+$], 305. Anal. Calcd for $C_{15}H_{14}N_4O_2$: C, 63.82; H, 5.00; N, 19.85. Found: C, 63.94; H, 4.86; N, 19.95.

4.7. Ethyl 6-amino-5-cyano-2-methyl-4-(3-pyridyl)-3-pyridinecarboxylate (25)

Following the General method, from compound **16**[13] (3.0 g, 10.50 mmol), ammonium acetate (8.17 g, 105.2 mmol), acetic acid (131 mL), after 24 h, compound **25** (450 mg, 15%) was obtained: mp 179–181 °C; IR (KBr) ν 3386, 3323, 3176, 2221, 1713, 1653, 1574, 1554, 1278, 1184 cm^{-1}; ^{1}H NMR (DMSO-d_6, 200 MHz) δ 8.67 (d, J = 4.6 Hz, 1H), 8.49 (s, 1H), 7.78 (d, J = 7.5 Hz, 1H), 7.53 (dd, J_1 = 4.6 Hz, J_2 = 7.5 Hz, 1H) 7.45 (s, 2H), 3.88 (q, J = 7.2 Hz, 2H), 2.42 (s, 3H), 0.77 (t, 3H); ^{13}C NMR (DMSO-d_6, 50.3 MHz) δ 166.4, 161.3, 159.8, 151.7, 150.4, 148.2, 136.0, 134.2, 123.7, 118.6, 116.2, 86.9, 61.1, 23.9, 13.7; MS (APCI+) m/z [M+1]$^+$ 283, [M+Na$^+$], 305. Anal. Calcd for $C_{15}H_{15}N_4O_2$: C, 63.82; H, 5.00; N, 19.82. Found: C, 63.58; H, 4.93; N, 18.58.

4.7.1. Ethyl 6-amino-5-cyano-2-methyl-4-(2-thienyl)-3-pyridinecarboxylate (26). Following the General method, from compound **17**[13] (2 g, 6.86 mmol), ammonium acetate (5.29 g, 68.6 mmol), acetic acid (85 mL), after 7 h, product 26 (360 mg, 5%) was obtained: mp 213–216 °C; IR (KBr) ν 3391, 3323, 3179, 2219, 1713, 1651, 1558, 1277, 1183, 1065 cm^{-1}; ^{1}H NMR (DMSO-d_6, 200 MHz) δ 7.84 (d, J = 4.9 Hz, 1H), 7.36 (s, 2H), 7.25 (d, J = 3.7 Hz, 1H), 7.19 (dd, J_1 = 3.7 Hz, J_2 = 4.9 Hz, 1H), 3.99 (q, J = 7.1 Hz, 2H), 2.36 (s, 3H), 0.94 (t, 3H); ^{13}C NMR (DMSO-d_6, 50.3 MHz) δ 167.5, 160.6, 160.4, 146.5, 135.8, 129.8, 129.7, 128.3, 118.8, 116.5, 87.7, 61.7, 23.8, 14.1; MS (APCI+) m/z [M+1]$^+$ 288, [M+Na$^+$] 310. Anal. Calcd for $C_{14}H_{13}N_3O_2S$: C, 57.92; H, 4.86; N, 9.65; S, 11.04. Found: C, 58.01; H, 4.59; N, 10.01; S, 11.25.

4.8. Ethyl 5-amino-4,6,7,8-tetrahydro-2-methyl-4-(4-pyridyl)-cyclopeta[*b*]pyran[3,2-*e*]pyridine-3-carboxylate (2)

Following the General method, from compound **15**[13] (219.5 mg, 0.77 mmol), $AlCl_3$ (148 mg, 1.12 mmol), $ClCH_2CH_2Cl$ (7.7 mL), cyclopentanone (99 μL, 1.12 mmol)], after 28 h, product **2** (175 mg, 65%) was obtained: mp 191–192 °C; IR (KBr) ν 3429, 3355, 2973, 1687, 1645, 1594, 1415, 1372, 1256, 1212, 1071 cm^{-1}; ^{1}H NMR (DMSO-d_6, 200 MHz) δ 8.39 (d, J = 5.9 Hz, 2H), 7.29 (d, J = 5.9 Hz, 2H), 5.93 (s, 2H), 5.03 (s, 1H), 4.05 (q, J = 6.7 Hz, 2H), 2.67 (m, 4H), 2.36 (s, 3H), 1.95 (m, 2H), 1.18 (t, 3H); ^{13}C NMR (DMSO-d_6, 50.3 MHz) δ 166.2, 161.3, 160.9, 156.5, 153.4, 149.7 (2C), 149.5, 123.5 (2C), 117.6, 106.4, 97.5, 60.4, 36.1, 33.9, 27.7, 22.1, 19.4, 14.3; MS (APCI+) m/z [M+1] 352.3. Anal. Calcd for $C_{20}H_{21}N_3O_3$: C, 68.36; H, 6.02; 11.96. Found: C, 68.25; H, 5.95; N, 11.64.

4.9. Ethyl 5-amino-6,7,8,9-tetrahydro-2-methyl-4-(4-pyridyl)-4*H*-pyran[2,3-*b*]quinoline-3-carboxylate (3)

Following the General method, from compound **15**[13] (100 mg, 0.35 mmol), $AlCl_3$ (70.67 mg, 0.52 mmol),

$ClCH_2CH_2Cl$ (5 mL), cyclohexanone (50 mg, 0.52 mmol), after 24 h, product **3** (109.2 mg, 85%) was obtained: mp 222–223 °C; IR (KBr) ν 3438, 3358, 3240, 2930, 1689, 1644, 1601, 1571, 1454, 1301, 1207, 1061 cm^{-1}; ^1H NMR (DMSO-d_6, 200 MHz) δ 8.39 (d, J = 5.8 Hz, 2H), 7.30 (d, J = 5.8 Hz, 2H), 5.77 (s, 2H), 5.05 (s, 1H), 4.05 (q, J = 7.0 Hz, 2H), 2.37 (s, 3H), 2.25 (m, 2H), 2.18 (m, 2H), 1.66 (m, 4H), 1.14 (t, 3H); ^{13}C NMR (DMSO-d_6, 50.3 MHz) δ 166.6, 161.3, 154.6, 153.8, 153.3, 151.7, 150.1 (2C), 123.9 (2C), 113.8, 106.5, 97.9, 60.8, 36.5, 32.7, 23.7, 22.9, 22.7, 19.8, 14.7; MS (APCI+) m/z $[M+1]^+$ 366.1; $[M+Na]^+$ 388.1; $[2M+Na]^+$ 754.1. Anal. Calcd for $C_{21}H_{23}N_3O_3$: C, 69.01; H, 6.35; N, 11.50. Found: C, 68.92; H, 6.30; N, 11.35.

4.10. Ethyl 5-amino-4,6,7,8,9,10-hexahydro-2-methyl-4-(4-pyridyl)-cyclohepta[*b*]pyran[3,2-*e*]pyridine-3-carboxylate (4)

Following the General method, from compound **15**[13] (100 mg, 0.35 mmol), $AlCl_3$ (70.67 mg, 0.52 mmol), $ClCH_2CH_2Cl$ (4 mL), cycloheptanone (138.62 mg, 0.35 mmol), after 26 h, compound **4** (120 mg, 97%) wasd obtained: mp 165–167 °C; IR (KBr) ν 3401, 3238, 2923, 2851, 1692, 1645, 1597, 1567, 1430, 1260, 1233, 1218 cm^{-1}; ^1H NMR (DMSO-d_6, 200 MHz) δ 8.39 (d, J = 5.7 Hz, 2H), 7.28 (d, J = 5.7 Hz, 2H), 5.75 (s, 2H), 5.02 (s, 1H), 4.05 (q, J = 7.0 Hz, 2H), 2.63 (m, 4H), 2.36 (s, 3H), 1.53 (m, 6H), 1.17 (t, 3H); ^{13}C NMR (DMSO-d_6, 50.3 MHz) δ 166.2, 160.7, 159.9, 153.9, 153.3, 150.7, 149.7 (2C), 123.5 (2C), 118.6, 106.2, 98.4, 60.3, 38.3, 36.5, 31.9, 27.1, 26.2, 25.1, 19.3, 14.3; MS (APCI+) m/z [M+1] 380.3, [2M+Na] 782.3. Anal. Calcd for $C_{22}H_{25}N_3O_3$: C, 69.62; H, 6.64; N, 11.08. Found: C, 69.68; H, 6.65; N, 11.02.

4.11. Ethyl 5-amino-4,6,7,8-tetrahydro-2-methyl-4-(3-pyridyl)-cyclopenta[*b*]pyran[3,2-*e*]pyridine-3-carboxylate (5)

Following the General method, from compound **16** (219.5 mg, 0.77 mmol), $AlCl_3$ (148 mg, 1.12 mmol), $ClCH_2CH_2Cl$ (7 mL), cyclopentanone (99 μL, 1.12 mmol), after 96 h, product **5** (203 mg, 73%) was obtained: mp 213–215 °C; IR (KBr) ν 3469, 2186, 2961, 1709, 1684, 1644, 1578, 1375, 1261, 1204 cm^{-1}; ^1H NMR (DMSO-d_6, 200 MHz) δ 8.62 (d, J = 1.7 Hz, 1H), 8.33 (dd, J = 1.7 Hz, J = 4.7 Hz, 1H), 7.58 (dd, J = 1.8 Hz, J = 8.2 Hz, 1H), 7.24 (dd, J = 1.7 Hz, J = 8.2 Hz, 1H), 5.96 (s, 2H), 5.03 (s, 1H), 4.07 (q, J = 6.7 Hz, 2H), 2.63 (m, 4H), 2.39 (s, 3H), 1.97 (m, 2H), 1.19 (t, 3H); ^{13}C NMR (DMSO-d_6, 50.3 MHz) δ 166.5, 161.3, 160.9, 156.7, 149.8, 149.5, 148.1, 140.7, 135.7, 124.0, 118.0, 106.8, 98.5, 60.6, 34.5, 34.2, 27.9, 22.3, 19.6, 14.5; MS (APCI+) m/z $[M+1]^+$ 352.0, $[M+Na]^+$ 374.0, $[2M+Na]^+$ 725.3. Anal. Calcd for $C_{20}H_{21}N_3O_3$: C, 68.36; H, 6.02; N, 11.96. Found: C, 68.12; H, 5.94; N, 11.75.

4.12. Ethyl 5-amino-6,7,8,9-tetrahydro-2-methyl-4-(3-pyridyl)-4*H*-pyran[2,3-*b*]quinoline-3-carboxylate (6)

Following the General method, from compound **16** (200 mg, 0.7 mmol), $AlCl_3$ (148 mg, 1.4 mmol), $ClCH_2CH_2Cl$ (6 mL), cyclohexanone (137 mg, 1.4 mmol)], after 36 h, product **6** (225 mg, 88%) was obtained: mp 176–178 °C; IR (KBr) ν 3436, 2932, 1685, 1643, 1572, 1455, 1302, 1571, 1227, 1209, 1099, 1063 cm^{-1}; ^1H NMR (DMSO-d_6, 200 MHz) δ 8.63 (d, J = 1.8 Hz, 1H), 8.33 (d, J = 4.6 Hz, 1H), 7.59 (d, J = 3.6 Hz, 1H), 7.25 (dd, J = 3.6 Hz, J = 4.7 Hz, 1H), 5.77 (s, 2H), 5.05 (s, 1H), 4.07 (q, J = 7.0 Hz, 2H), 2.39 (s, 3H), 2.31 (m, 2H), 2.19 (m, 2H), 1.68 (m, 4H), 1.15 (t, 3H); ^{13}C NMR (DMSO-d_6, 50.3 MHz) δ 166.6, 160.6, 154.2, 152.8, 151.2, 149.6, 147.8, 140.4, 135.5, 123.8, 113.4, 106.6, 98.0, 60.8, 34.3, 32.3, 23.3, 22.5, 22.3, 19.4, 14.7; MS (APCI+) m/z $[M+1]^+$ 366.0, $[M+Na]^+$ 388.1, $[2M+Na]^+$ 753.1. Anal. Calcd for $C_{21}H_{23}N_3O_3$: C, 69.01; H, 6.35; N, 11.50. Found: C, 68.80; H, 6.50; N, 11.27.

4.13. Ethyl 5-amino-4,6,7,8,9,10-hexahydro-2-methyl-4-(3-pyridyl)-cyclohepta[*b*]pyran[3,2-*e*]pyridine-3-carboxylate (7)

Following the General method, from compound **16** (100 mg, 0.35 mmol), $AlCl_3$ (70.61 mg, 0.52 mmol), $ClCH_2CH_2Cl$ (4 mL), cycloheptanone (58.32 μL, 0.52 mmol), after 24 h, product **7** (102 mg, 77%) was obtained: mp 180–182 °C; IR (KBr) ν 3459, 3372, 2922, 2849, 1689, 1640, 1565, 1428, 1260, 1214 cm^{-1}; ^1H NMR (DMSO-d_6, 200 MHz) δ 8.63 (s, 1H), 8.31 (d, J = 3.8 Hz, 1H), 7.56 (d, J = Hz, 1H), 7.23 [dd, J = 3.8 Hz, J = 7.8 Hz, 1H), 5.77 (s, 2H), 5.00 (s, 1H), 4.05 (q, J = 6.9 Hz, 2H), 2.72 (m, 4H), 2.36 (s, 3H), 1.72 (m, 6H), 1.17 (t, 3H); ^{13}C NMR (DMSO-d_6, 50.3 MHz) δ 166.2 (C=O), 160.4 (C11a), 159.8 (C2), 153.8 (C10a), 150.5, 149.7, 147.8, 140.4, 135.6, 123.8, 118.6, 106.5, 98.8, 60.3, 38.2, 34.5, 32.0, 27.2, 26.2, 25.0, 19.3, 14.3; MS (APCI+) m/z [M+1] 380.0, $[M+Na]^+$ 402.0, [2M+Na] 781.0. Anal. Calcd for $C_{22}H_{25}N_3O_3$: C, 69.62; H, 6.64; N, 11.08. Found: C, 69.54; H, 6.81; N, 11.13.

4.14. Ethyl 5-amino-4,6,7,8,9,10-hexahydro-2-methyl-4-(2-thienyl)cyclohepta[*b*]pyran[3,2-*e*]pyridine-3-carboxylate (8)

Following the General method, from compound **17**[13] (200 mg, 0.69 mmol), $AlCl_3$ (138 mg, 1.03 mmol), $ClCH_2CH_2Cl$ (7 mL), cyclopentanone (247 μL, 2.89 mmol), after 96 h, product **8** (70 mg, 29%) was obtained: mp 178–179 °C; IR (KBr) ν 3413, 3350, 3205, 2925, 2851, 1691, 1652, 1371, 1253, 1210 cm^{-1}; ^1H NMR (DMSO-d_6, 200 MHz) δ 7.24 (d, J = 4.3 Hz, 1H), 6.96 (d, J = 4.0 Hz, 1H), 6.85 (dd, J = 4.3 Hz, J = 4.0 Hz, 1H), 5.99 (s, 2H), 5.39 (s, 1H), 4.15 (q, J = 7.6 Hz, 2H), 2.65 (m, 2H), 2.51 (s, 3H), 1.99 (m, 2H), 1.34 (m, 2H), 1.28 (t, 3H); ^{13}C NMR (DMSO-d_6, 50.3 MHz) δ 166.0, 160.6, 159.9, 156.2, 149.1, 148.7, 126.2, 124.6, 124.2, 117.1, 107.0, 98.3, 60.1, 33.6, 31.5, 27.4, 21.8, 19.0, 14.1; MS (APCI+) m/z $[M+1]^+$ 357, $[M+Na]^+$ 379.2, $[2M+Na]^+$ 735.3. Anal. Calcd for $C_{19}H_{20}N_2O_3S$: C, 64.02; H, 5.66; N, 7.86; S, 8.98. Found: C, 63.89; H, 5.80; N, 7.77; S, 8.98.

4.15. Ethyl 5-amino-6,7,8,9-tetrahydro-2-methyl-4-(2-thienyl)-4*H*-pyran[2,3-*b*] quinoline-3-carboxylate (9)

Following the General method, from compound **17**[13] (200 mg, 0.69 mmol), $AlCl_3$ (138 mg, 1.04 mmol), $ClCH_2CH_2Cl$ (7 mL), cyclohexanone (292 μL, 2.89 mmol), after 1.5 h, product **9** (210 mg, 83%) was obtained: mp 204–205 °C; IR (KBr) *v* 3440, 2933, 1691, 1645, 1603, 1572, 1455, 1302, 1229, 1206, 1100, 1061 cm^{-1}; ^1H NMR (DMSO-d_6, 200 MHz) δ 7.24 (d, J = 5.0 Hz, 1H), 6.97 (d, J = 4.7 Hz, 1H), 6.84 (dd, J = 5.0 Hz, J = 4.7 Hz, 1H), 5.83 (s, 2H), 5.40 (s, 1H), 4.15 (q, J = 7.0 Hz, 2H), 2.55 (m, 2H), 2.35 (s, 3H), 2.26 (m, 2H), 1.71 (m, 4H), 1.24 (t, 3H); ^{13}C NMR (DMSO-d_6, 50.3 MHz) δ 166.8, 160.5, 154.6, 153.1, 151.8, 149.3, 126.9, 125.5, 125.0, 113.6, 107.7, 98.8, 60.8, 32.7, 32.3, 23.7, 23.0, 22.8, 19.8, 14.8; MS (APCI+) *m*/*z* $[M+1]^+$ 371, $[2M+Na]^+$ 763.3. Anal. Calcd for $C_{20}H_{22}N_2O_3S$: C, 64.84; H, 5.99; N, 7.56; S, 8.66. Found: C, 64.65; H, 6.10; N, 7.58; S, 8.90.

4.16. Ethyl 5-amino-4,6,7,8,9,10-hexahydro-2-methyl-4-(2-thienyl)cyclohepta[*b*]pyran[3,2-*e*]pyridine-3-carboxylate (10)

Following the General method, from compound **17**[13] (200 mg, 0.69 mmol), $AlCl_3$ (138 mg, 1.03 mmol), $ClCH_2CH_2Cl$ (7 mL), cycloheptanone (350 μL, 2.90 mmol), after 4 h, product **10** (160 mg, 61%) was obtained: mp 155–157 °C; IR (KBr) *v* 3384, 2919, 2849, 1688, 1624, 1564, 1371, 1262, 1208 cm^{-1}; ^1H NMR (DMSO-d_6, 200 MHz) δ 7.25 (d, J = 5.1 Hz, 1H), 6.95 (d, J = 4.1 Hz, 1H), 6.84 (dd, J = 5.1 Hz, J = 4.1 Hz, 1H), 5.82 (s, 2H), 5.37 (s, 1H), 4.15 (q, J = 7.0 Hz, 2H), 2.52 (m, 4H), 2.24 (s, 3H), 1.46 (m, 6H), 1.26 (t, 3H); ^{13}C NMR (DMSO-d_6, 50.3 MHz) δ 166.4, 160.1, 153.8, 150.7, 148.9, 147.8, 127.3, 126.6, 125.8, 116.1, 107.2, 99.2, 60.3, 38.1, 32.1, 27.3, 26.3, 25.1, 19.4, 18.3, 14.4; MS (APCI+) *m*/*z* $[M+1]^+$ 385.1, $[2M+Na]^+$ 791.5. Anal. Calcd for $C_{21}H_{24}N_2O_3S$: C, 65.60; H, 6.29; N, 7.29; S, 8.34. Found: C, 65.39; H, 6.50; N, 7.45; S, 8.60.

4.17. Ethyl 4-(1-acetyl-1*H*-pyrrol-2-yl)-5-amino-6,7,8,9-tetrahydro-2-methyl-4*H*-pyran[2,3-*b*]quinoline-3-carboxylate (11)

Following the General method, from compound **18** (200 mg, 0.64 mmol), $AlCl_3$ (127.68 mg, 0.96 mmol), $ClCH_2CH_2Cl$ (6 mL), cyclohexanone (133 μL, 1.28 mmol), after 3 h, product **11** (110 mg, 43%) was obtained: mp 172–174 °C; IR (KBr) *v* 3458, 3347, 3246, 2958, 2930, 1710, 1635, 1572, 1455, 1367, 1302, 1218, 1061, 947, 726, 641 cm^{-1}; ^1H NMR (DMSO-d_6, 200 MHz) δ 7.16 (d, J = 3.2 Hz, 1H), 6.05 (t, J = 3.4 Hz, 1H), 5.85 (dd, J = 3.2 Hz, J = 3.4 Hz, 1H), 5.75 (s, 2H), 5.55 (s, 1H), 3.88 (q, J = 7.0 Hz, 2H), 2.57 (s, 3H), 2.22 (s, 3H), 2.16 (m, 2H), 1.60 (m, 6H), 0.98 (t, 3H); ^{13}C NMR (DMSO-d_6, 50.3 MHz) δ 173.4, 166.4, 159.4, 153.9, 152.4, 151.3, 140.9, 121.7, 115.3, 112.9, 111.9, 107.8, 98.7, 60.1, 32.2, 29.2, 25.0, 23.3, 22.6, 22.4, 19.4, 14.3; MS (APCI+) *m*/*z* $[M+1]^+$ 396.3; $[2M+Na]^+$ 813.5. Anal. Calcd for $C_{22}H_{25}N_3O_4$: C, 66.82; H, 6.37; N, 10.63. Found: C, 67.00; H, 6.60; N, 10.61.

4.18. Ethyl 5-amino-7,8-dihydro-2-methyl-4-(4-pyridyl)-6*H*-cyclopenta[*b*][1,8]naphthyridine-3-carboxylate (12)

Following the General method, from compound **19** (150 mg, 0.53 mmol), $AlCl_3$ (105.3 mg, 0.79 mmol), $ClCH_2CH_2Cl$ (5 mL), cyclopentanone (72 μL, 0.79 mmol), after 18 h, product **12** (118 mg, 61%) was obtained: mp 245–247 °C; IR (KBr) *v* 3492, 3435, 3195, 2956, 1724, 1630, 1593, 1550, 141, 1371, 1271, 1087, 592 cm^{-1}; ^1H NMR (DMSO-d_6, 200 MHz) δ 8.64 (d, J = 4.3 Hz, 2H), 7.35 (d, J = 4.3 Hz, 2H), 4.96 (s, 2H), 3.84 (q, J = 7.1 Hz, 2H), 2.85 (m, 2H), 2.59 (m, 2H), 2.46 (s, 3H), 1.96 (m, 2H), 0.77 (t, 3H); ^{13}C NMR (DMSO-d_6, 50.3 MHz) δ 170.6, 167.4, 156.7, 154.7, 150.1 (2C), 147.5, 145.4, 142.7, 125.4, 124.1 (2C), 116.8, 106.1, 61.5, 35.1, 28.1, 23.4, 22.0, 13.6; MS (APCI+) *m*/*z* $[M+1]^+$ 349.2, $[2M+Na]^+$ 719.5. Anal. Calcd for $C_{20}H_{20}N_4O_2$: C, 68.95; H, 5.79; N, 16.08. Found: C, 69.00; H, 5.93; N, 16.34.

4.19. Ethyl 5-amino-6,7,8,9-tetrahydro-2-methyl-4-(4-pyridyl)-benzo[*b*][1–8]naphthyridine-3-carboxylate (13)

Following the General method, from compound **19** (120 mg, 0.42 mmol), $AlCl_3$ (84 mg, 0.63 mmol), $ClCH_2CH_2Cl$ (4 mL), cyclohexanone (66 μL, 0.63 mmol), after 40 h, product **l3** (75 mg, 49%) was obtained: mp 208–210 °C; IR (KBr) *v* 3498, 3334, 3230, 2931, 1725, 1629, 1571, 1540, 1445, 1269, 1223 cm^{-1}; ^1H NMR (DMSO-d_6, 200 MHz) δ 8.76 (d, J = 5.9 Hz, 2H), 7.48 (d, J = 5.9 Hz, 2H), 5.28 (s, 2H), 3.96 (q, J = 7.0 Hz, 2H), 2.87 (m, 2H), 2.52 (s, 3H), 2.36 (m, 2H), 1.81 (m, 4H), 0.88 (t, 3H); ^{13}C NMR (DMSO-d_6, 50.3 MHz) δ 167.4, 161.1, 155.8, 153.7, 150.6, 150.4 (2C), 145.4, 142.7, 126.0, 124.2 (2C), 112.2, 105.6, 61.5, 33.5, 23.9, 23.6, 22.4 (2C), 13.8; MS (APCI+) *m*/*z* $[M+1]^+$ 363.0; $[M+Na]^+$ 385.0; $[2M+Na]^+$ 749.0. Anal. Calcd for $C_{21}H_{23}N_4O_2$: C, 69.59; H, 6.12; N, 15.46. Found: C, 69.31; H, 6.30; N, 15.28.

4.20. Ethyl 5-amino-7,8,9,10-tetrahydro-2-methyl-4-(4-pyridyl)-6*H*-cyclohepta[*b*][1,8]naphthyridine-3-carboxylate (14)

Following the General method, from compound **19** (150 mg, 0.53 mmol), $AlCl_3$ (106 mg, 0.80 mmol), $ClCH_2CH_2Cl$ (5 mL), cycloheptanone (95 μL, 0.8 mmol), after 4 h, product **14** (184 mg, 92%) was obtained: mp 210–212 °C; IR (KBr) *v* 3477, 3366, 3032, 2925, 2856, 1731, 1638, 1568, 1544, 1272, 1224, 1084 cm^{-1}; ^1H NMR (DMSO-d_6, 200 MHz) δ 8.63 (d, J = 5.7 Hz, 2H), 7.37 (d, J = 5.7 Hz, 2H), 5.19 (s, 2H), 3.85 (q, J = 7.0 Hz, 2H), 2.54 (m, 4H), 2.45 (s, 3H), 1.62 (m, 6H), 0.77 (t, 3H); ^{13}C NMR (DMSO-d_6, 50.3 MHz) δ 167.6, 167.3, 155.2, 153.0, 150.1 (2C), 149.2, 145.3, 142.6, 126.2, 124.1 (2C), 117.2, 106.5, 61.6, 39.0, 31.6, 27.3, 26.5, 25.3, 23.3, 13.6; MS (APCI+) *m*/*z* $[M+1]^+$ 377.2, $[M+Na]^+$ 399.22, $[2M+Na]^+$ 775.7. Anal. Calcd for $C_{22}H_{24}N_4O_2$: C, 70.19; H, 6.43; N, 14.88. Found: C, 70.35; H, 6.76; N, 15.16.

4.21. Biological studies

4.21.1. AChE activity. To measure the AChE inhibitory activity of the compounds, we followed the spectrometric method of Rappaport et al.[17] using AChE from electric eel (*Torpedo californica*) and acetylcholine chloride (29.5 mM) as a substrate. The reaction took place in a final volume of 2.5 mL of an aqueous solution containing 0.78 U of AChE and 1.9 mM *m*-nitrophenol to produce a yellow colour, which is lost as a function of the enzymatic activity. Inhibition curves were obtained by incubating with the different compounds for 30 min; a control was always present to determine the 100% value for the enzymatic activity. After the 30 min incubation, the consumption of *m*-nitrophenol was determined by measuring absorbance at 405 nm in a spectrophotometric plate reader (iEMS Reader MF, Labsystems). The concentration of compound, which produced 50% inhibition of the AChE activity (IC_{50}) was calculated by transforming the values of absorbance to Rappaport enzymatic activity units, extrapolating from a calibration curve obtained previously. Data are means ± s.e.m of at least three different experiments in triplicate.

4.22. Chromaffin cells

Bovine adrenomedullary chromaffin cells were isolated following standard methods[20] with some modifications.[21] Cells were suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% foetal calf serum, 10 μM cytosine arabinoside, 10 μM fluorodeoxyuridine, 50 IU mL^{-1} penicillin and 50 μg mL^{-1} streptomycin. Cells were plated in 24-multiwell Costar plates and were used 1–5 days after plating. Medium was replaced after 24 h and again after 2–3 days.

Lactic dehydrogenase (LDH) activity. To study the cytoprotective action of the new compounds against veratridine-induced cell death, drugs were given at time zero ($t = 0$) and maintained for 48 h; at $t = 24$ h veratridine was added to the médium in the presence of the drugs; then, at $t = 48$ h, cell viability was assessed measuring lactic dehydrogenase (LDH) activity. Extracellular and intracellular LDH activity was spectrophotometrically measured using a Cytotoxicity Cell Death kit (Roche-Boehringer Mannheim), according to manufacturer's instructions. Total LDH activity was defined as the sum of intracellular and extracellular LDH activity; released LDH was defined as the percentage of extracellular activity compared to total LDH activity.

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