JOURNAL OF
MEDICINAL
CHEMISTRY

Volume 36, Number 11 May 28, 1993

Articles

Synthesis and Amnesia-Reversal Activity of a Series of 7- and 5-Membered 3-Acylamino Lactams†

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Received June 25, 1992

A series of 3-(acylamino)-ε-caprolactams and 3-(acylamino)-2-pyrrolidinones was synthesized. Some of these compounds reversed at different degrees electroconvulsive shock- and Scopolamine-induced amnesia, using a step-through passive avoidance in mice. Classical nootropic drugs, i.e., Aniracetam, Oxiracetam, and Piracetam, were used as reference compounds. Within the analyses of data performed, we introduced a new parameter, the confrontation index (CI), which is a function of Mann-Whitney's *U* statistic. The CI permits a common scale of activity of substances to be generated, independently of probabilistic hypotheses, with higher scores representing higher activities. The most active compounds were characterized by the formylamino and [3-(trifluoromethyl)benzoyl]amino groups in the 3-position of the ring. None of the substances assayed showed any effect on spontaneous behavior and neurovegetative system.

Over the last two decades, an ever growing interest has been devoted to the search for new drugs that might be useful for prevention or treatment of human cognitive disorders. Although cognitive impairments may occur in people of any age, the increase in life expectancy has made treatment of age-related cognitive disorders one of today's scientific, medical, and social targets.

Cognition enhancing agents include a wide range of different substances,[1,2] characterized by different and, so far, not fully understood mechanisms of action.

In this context, nootropic drugs are substances known to be able to ameliorate cognitive functions by acting directly and selectively on the highest telencephalic integrative activity. The absence of the pharmacological effects usually exerted by neuropsychotropic drugs and low toxicity are among the major characteristics of these substances which have also been shown to enhance resistance to brain insults.[3] The most representative nootropic agents are those belonging to the class of 2-pyrrolidinones, such as Piracetam (**1**), Oxiracetam (**2**), Pramiracetam (**3**), Aniracetam (**4**), Rolziracetam (**5**), etc.[4]

It has recently been reported that some amino acid derivatives, for example (*Z*)-prolylprolynal (**6**),[5] SUAMM 1221 (**7**),[6] and [(4-phenylbutanoyl)prolyl]prolynal (**8**)[7] possess antiamnesic properties and the ability to strongly

† This paper has been presented in part at the Congresso Interdivisionale della Società Chimica Italiana, San Benedetto del Tronto, Italy, September 30–October 5, 1990, Abstr, p 115, and at the Fourth Meeting of the Italian Society of Neuroscience, Palermo, Italy, 4–5 December 1990, Abstr. *Neurosci. Lett.* **1990**, *39*, S62.

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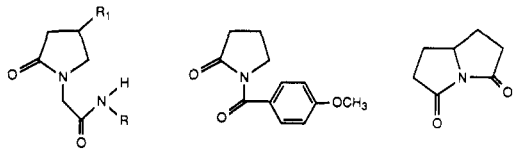
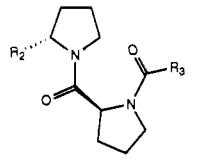


4 5

1: R = H, R_1 = H
2: R = H, R_1 = OH
3: R = $CH_2CH_2N[CH(CH_3)_2]_2$, R_1 = H



6: R_2 = CHO, R_3 = OCH_2Ph
7: R_2 = H, R_3 = $(CH_2)_3Ph$
8: R_2 = CHO R_3 = $(CH_2)_3Ph$

inhibit mammalian prolyl endopeptidase (PEP). Also, some nootropics such as Aniracetam and Pramiracetam have also been found to inhibit brain PEP,[8] suggesting the hypothesis of a common mechanism of action. In fact, PEP inhibitors and nootropics might influence memory by prolonging the lifetime of endogenous neuropeptides that modulate cholinergic neurotransmission.[9]

In a previous study, the conformational features of 10 known amnesia-reversal compounds and two potent PEP inhibitors showing strong antiamnesic activity have been analyzed by molecular mechanics calculations[10] in order to identify a common spatial disposition of the polar functional groups present in all the molecules (an NC=O amidic group and an XC=O group, with X = O, N). These polar groups, included in the pharmacophore, are supposed to be involved in the biological interaction.

Minimum-energy conformations of investigated compounds were calculated by the MM2 force field.[11] Principal component analysis (PCA) led to the identification of three interatomic distances (D_{O-O}, D_{C-C}, and D_{N-X}) which provide all the information necessary to describe the relative spatial disposition of the two functional groups.

Cluster analysis was then performed to group minimum-energy conformations according to the values of those three distances. Clusters were checked to single out the ones containing conformations of the maximum number of active compounds. This procedure allowed us to point out two acceptable pharmacophore models, characterized by the distance values 3.57, 3.14, and 3.07 Å, and by 3.65, 3.24, and 3.75 Å, respectively.

Based on the hypothesis that common stereoelectronic factors produce a similar biological behavior, we sought for structures having a spatial disposition of polar functional groups according to the pharmacophore models and found that 3-(acylamino)-ε-caprolactam and, to a lesser extent, 3-(acylamino)-2-pyrrolidinone presented a satisfactory fitting. These findings prompted us to synthesize a series of (*R*)- and (*S*)-3-(acylamino)-ε-caprolactams and (*S*)-3-(acylamino)-2-pyrrolidinones, derivatized at will at the nitrogen atom of the ring, in order to evaluate their antiamnesic activity in the electroconvulsive shock (ECS)- and Scopolamine-induced amnesia models in mice.

Chemistry

Derivatives **11–22** (Table I) were readily synthesized, as outlined in Scheme I, from **9A** ((*S*)-3-amino-ε-caprolactam), **9B** ((*R*)-3-amino-ε-caprolactam), **10** ((*S*)-3-amino-2-pyrrolidinone), and the relative carboxylic acids, using carbonyldiimidazole (CDI) or 2-ethoxy-1-(ethoxycarbonyl)-1,2-dihydroquinoline (EEDQ) as condensing agents. As an alternative, the relative acyl chloride was used.

All the other compounds, substituted at the nitrogen atom of the lactam (Table II), were obtained as outlined in Scheme II.

(Acylamino)-ε-caprolactams **11** and **20** were alkylated with $BrCH_2COOMe$ and NaH to give the methyl esters **24** and **27**, which were reduced to give the corresponding alcohols **25** and **28**, respectively. Amides **23** and **26** were obtained by bubbling gaseous NH_3 into the MeOH solution of **24** and **27**, respectively. Compound **30** was obtained by hydrolysis of the intermediate dimethyl acetal **29**, which was obtained by reacting compound **20** with $BrCH_2CH(OMe)_2$ and KH.

Compounds **11** and **12** are chemically known substances.[12,13]

Molecular Modeling

A molecular modeling study was performed in order to verify whether different candidate compounds might fit the pharmacophore models previously defined.[10] For each compound, minimum-energy conformations were calculated by a systematic search for the accessible conformational space. Analysis of the three distance D_{O-O}, D_{C-C}, and D_{N-X} values in the conformational minima pointed out that 3-(acylamino)-ε-caprolactams (**12** for instance) and, to a lesser extent, 3-(acylamino)-2-pyrrolidinones (**22** for instance) presented a satisfactory fitting to both the pharmacophore models.

Table III reports the relative energies, with respect the global minima and the pharmacophore distance values for the most interesting conformations of compounds **12** and **22**. These conformations were selected because, as regards the six atoms of the pharmacophore, they provide the best fitting to the "active conformations" of compound **8** possessing the *S* configuration.[10] The goodness of the fit is reported in the table as the root mean square (RMS) value.

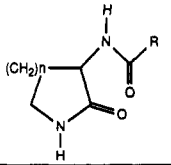
Pharmacology

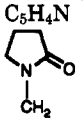
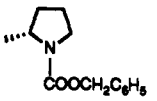
The most commonly used screening method to investigate the effect of nootropic drugs consists of behavioral tests on learning and memory in which the amnesia-reversal activity (AA) is evaluated in relation to amnesic treatments, such as Scopolamine,[14] ECS,[15] or hypoxia.[3] In this study, amnesia was induced in mice by either ECS or Scopolamine administration, and AA of the various substances was determined by a step-through passive avoidance. The two different amnesic models were chosen to investigate for each substance an effect related to the cholinergic system (i.e., against Scopolamine administration[16]) or a general protective effect on CNS (i.e., against ECS treatment[17]).

Piracetam, Aniracetam, and Oxiracetam, which are considered to be major enhancers of cognitive functions among 2-pyrrolidinones derivatives, were used as reference compounds.

In order to find possible low-dose nootropic effects in our compounds, they were tested at the low dose–effect levels where the reference drugs have been reported to be scarcely effective.[14,15,18–20] This choice was made because nootropic drugs should be given chronically and it would

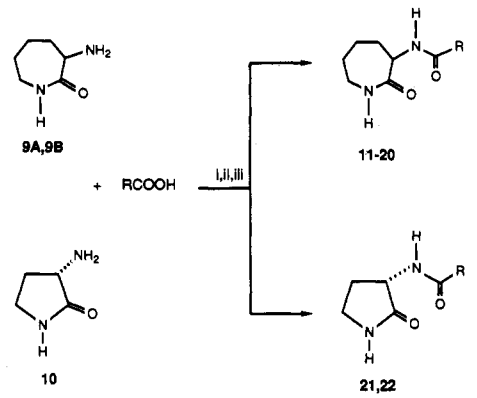
Table I. (*S*)-3-Acylamino Lactams (except 14, *R* Form)



no.	*n*	R	method	mp, °C	yield,[a] %	formula	anal.	$[\alpha]^{25}_{D}$[b]
11	3	H	A[c]	176	47	$C_7H_{12}N_2O_2$	C,H,N	+83[d]
12	3	CH_3	A[e]	147–149	62	$C_8H_{14}N_2O_2$	C,H,N	+13.7
13	3	3-$CF_3C_6H_4$	A	218–219	74	$C_{14}H_{15}F_3N_2O_2$	C,H,F,N	+26.9
14[f]	3	3-$CF_3C_6H_4$	A	218–219	74	$C_{14}H_{15}F_3N_2O_2$	C,H,F,N	−26.3
15	3	4-$OCH_3C_6H_4$	A	170–172	50	$C_{14}H_{18}N_2O_3$	C,H,N	+42.2
16	3	3-$(OCH_3)_3C_6H_2$	A	95–97	51	$C_{16}H_{22}N_2O_5$	C,H,N	+35.7
17	3	C_5H_4N	B	177	57	$C_{12}H_{15}N_3O_2$	C,H,N	+88[d]
18	3		A	245–247[g]	50	$C_{12}H_{19}N_3O_3$	C,H,N	+7.1
19	3		B	156–158	70	$C_{19}H_{25}N_3O_4$	C,H,N	−69.7
20	3	$(CH_2)_3C_6H_5$	A	106–108	90	$C_{16}H_{22}N_2O_2$	C,H,N	+10.2
21	1	3-$CF_3C_6H_4$	A	133–135	40	$C_{12}H_{11}F_3N_2O_2$	C,H,F,N	−31.5[h]
22	1	H	A	177–179	44	$C_5H_8N_2O_2$	C,H,N	−86.25[h]

[a] Yields were not optimized. [b] (c = 1% MeOH.) [c] Reference 12. [d] (c = 1% $CHCl_3$.) [e] Reference 13. [f] *R* form. [g] Crystallized from CH_3CN. [h] (c = 0.5% MeOH.)

Scheme I[a]



[a] (i) CDI, method A; (ii) EEDQ, method B; (iii) RCOOH → RCOCl, method C. **9A** = *S* form. **9B** = *R* form.

be important to restrict the amount of active principle, so as to minimize side effects. To confirm the validity of the testing procedure, here we report also the results of tests performed in the ECS model using the reference compounds at a dose level which enabled a significant reversal amnesia to be obtained.

Neuropharmacological tests in mice were also performed to see that the new compounds had no sedative, analgesic, or other effects on CNS. Their tolerability was also ascertained.

Data from the passive avoidance tests were analyzed through nonparametric statistical inferences. Furthermore, a novel parameter was introduced, the confrontation index (CI),[21] which is a function of *U* statistic and permits a common scale of activity of substances to be generated, with higher scores representing higher activities.

Lastly, the most active new substances, the standard compounds, as well as the *S* and *R* forms of the original 3-amino-ε-caprolactam, were evaluated for the ability to inhibit in vitro acetyl cholinesterase activity.

Results and Discussion

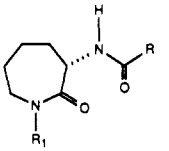
As expected, amnesia induced by either ECS or Scopolamine decreased retention latency of step-through passive avoidance in mice. Inferential statistical analysis revealed for each experiment significant differences between Sham and the respective control groups (Mann-Whitney *U* two-tailed test, confidence interval with $p < 0.05$ at least). Latencies of Sham and control groups from each experiment are reported as a whole data set in Table IV, for both ECS- and Scopolamine-induced amnesia models. The resulting overall CI for Sham versus control groups was 68 and 75% for the ECS and the Scopolamine models, respectively.

Table IV reports the efficacy of the substances. Compound **25** significantly reversed ECS-induced amnesia ($p < 0.05$; AA = 61) in the equimolar dose of 10 mg/kg of Piracetam, whereas compound **13** significantly reversed Scopolamine-induced amnesia ($p < 0.001$; AA = 65) in the equimolar dose of 1 mg/kg of Piracetam. Strikingly, compound **11** significantly reversed both ECS- ($p < 0.01$; AA = 71) and Scopolamine-induced amnesia ($p < 0.05$; AA = 49) in equimolar doses of 1 mg/kg of Piracetam. Relevant effects tending to statistical significance were exhibited by other tested substances, such as compounds **21** (AA = 58), **23** (AA = 55), **22** (AA = 53), **17** (AA = 50), **16** (AA = 42), **15** (AA = 37), **9A** (AA = 37), in the ECS model; and **9A** (AA = 47), **9B** (AA = 44), **12** (AA = 39), in the Scopolamine model. In Figure 1, the activity of each compound in both amnesic treatments is summarized graphically.

In accord with several authors,[14,15,18-20] the reference compounds used were not effective in reversing ECS- or Scopolamine-induced amnesia at the low dose levels used. However, the respective AA values reveal some activities of those substances. On the other hand, the notable activity exerted by Aniracetam ($p < 0.05$; AA = 83) and Oxiracetam ($p < 0.05$; AA = 57) at the equimolar dose of 100 mg/kg of Piracetam in the ECS model allow us to assert the validity of our experimental paradigm.

In general, AA values contribute to better evaluate the effect of a substance through the construction of scales of activities. Variations in the ring size suggested that 5-membered compounds were less active than 7-membered

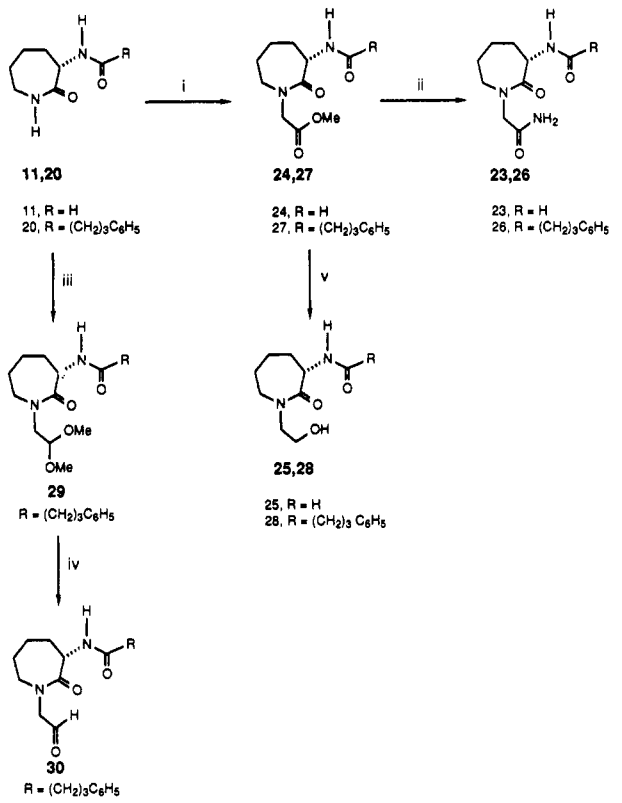
Table II. (*S*)-3-(Acylamino)-ε-caprolactam Derivatives



no.	R	R_1	mp, °C	yield, %	formula	anal.	$[\alpha]^{25}_D$
23	H	CH_2CONH_2	208–210	80	$C_9H_{15}N_3O_3$	C,H,N	+12.4[a]
24	H	CH_2COOCH_3	60–62	70	$C_{10}H_{16}N_2O_4$	C,H,N	+1.2[b]
25	H	CH_2CH_2OH	89–92	55	$C_9H_{16}N_2O_3$	C,H,N	+6.5[a]
26	$(CH_2)_3C_6H_5$	CH_2CONH_2	153–155	85	$C_{18}H_{25}N_3O_3$	C,H,N	+8.2[a]
27	$(CH_2)_3C_6H_5$	CH_2COOCH_3	*c*	74	$C_{19}H_{26}N_2O_4$	C,H,N	−9.7[b]
28	$(CH_2)_3C_6H_5$	CH_2CH_2OH	*c*	57	$C_{18}H_{26}N_2O_3$	C,H,N	−1[d]
30	$(CH_2)_3C_6H_5$	CH_2CHO	*c*	35	$C_{18}H_{24}N_2O_3$	C,H,N	+0.9[a]

[a] (*c* = 1% $CHCl_3$.) [b] (*c* = 1% MeOH.) [c] Not determined, oil. [d] (*c* = 2.2% MeOH.)

Scheme II[a]



[a] (i) $BrCH_2COOMe$, NaH, CH_3CN; (ii) NH_3, MeOH; (iii) $BrCH_2CH(OMe)_2$, KH, THF; (iv) CF_3COOH, H_2O/CH_3CN; (v) $NaBH_4$, $(CH_3)_3COH$, MeOH.

Table III. Relative Energies (ΔE, kcal mol^{-1}) and Interatomic Distances D_{O-O}, D_{C-C}, D_{N-X} (Å) for Conformations of Compounds **12** and **22** Presenting the Lowest RMS Values for the Fitting with the "Active Conformations" of Compound **8**[a]

compd	ΔE	D_{O-O}	D_{C-C}	D_{N-X}	RMS	pharm model
8	0.00	3.61	3.15	3.13		1
	2.69	3.52	3.22	3.79		2
12	1.65	3.22	3.04	3.23	0.14	1
	1.43	3.83	3.32	3.79	0.15	2
22	0.78	4.15	3.53	3.23	0.76	1
	0.92	3.46	3.11	3.61	0.16	2
pharm mod 1		3.57	3.14	3.07		
pharm mod 2		3.65	3.24	3.75		

[a] For comparison, the pharmacophore distance values of the active conformations of compound **8** and of the two pharmacophore models are reported.

ones. In fact, (*S*)-3-amino-2-pyrrolidinone **10** was less active than 3-amino-ε-caprolactams **9A** (*S* form) and **9B** (*R* form), especially in the Scopolamine-induced amnesia reversal test. Compound **9A** was a little more active than **9B** in the ECS test and almost equipotent in the Scopolamine test. (*S*)-3-(Formylamino)-ε-caprolactam **11** was more potent than (*S*)-3-(formylamino)-2-pyrrolidinone **22** in both ECS and Scopolamine tests. Furthermore, 3-[(trifluoromethyl)benzoyl]-ε-caprolactams **13** (*S* form) and **14** (*R* form) were more active than the 5-membered ring analogue **21** in the Scopolamine test. By contrast, **13** and **14** exhibited a lower activity compared with **21** in the ECS test.

Alkylation of N-1 atom with the hydroxyethyl group retained (**25** versus **11**) or enhanced (**28** versus **20**) ECS-induced amnesia reversal activity. On the contrary, in the Scopolamine test **11** was markedly more active than **25**, whereas no changes in activity were observed between **28** and **20**. The formylmethyl substitution gave a more potent compound in both ECS and Scopolamine tests (**30** compared with **20**), whereas (aminocarbonyl)methyl and (methoxycarbonyl)methyl substituents did not improve the activity in (3-phenylbutanoyl)amino derivatives (**26**, **27** versus **20**), and lowered the antiamnesic activity in 3-formylamino derivatives (**23**, **24** versus **11**).

Acylation of **9A** with aromatic acids either maintained or enhanced the activity of compounds **15**, **16**, **17**, only in the ECS test, whereas the activity of compound **13** was enhanced only in the Scopolamine test.

The amnesic effects of Scopolamine have been attributed to a central cholinergic action.[14,16] Thence, those 3-amino-ε-caprolactam derivatives (e.g., compounds **11** and **13**, and with slightly lower effects, **9A** and **9B**) that antagonized Scopolamine-induced amnesia might be suspected to exert some action on the cholinergic system.

Substances presenting the highest amnesia-reversal activity (**11**, **21**, **22**, **25**), the *S* and *R* forms of the original 3-amino-ε-caprolactam (**9A** and **9B**, respectively), and the standard compounds (Aniracetam, Oxiracetam, and Piracetam) did not inhibit AchE activity. These results are in accord with the inefficacy on AchE, as repeatedly reported for standard nootropic drugs.[22] In this context, compound **13** was not tested, although it was highly active in the Scopolamine model, because it could not be solubilized at the required experimental conditions. Further investigations are planned for the most active compounds, such as an evaluation of their effects on N-methyl-D-aspartate (NMDA) receptors, long term potentiation (LTP), etc., taking in account that some of these compounds have some structural features (glicinamide moiety) in common with substances known to involve effects on NMDA receptors.[23,24]

Table IV. Amnesia-Reversal Activity (AA) of New 7- and 5-Membered 3-Acylamino Lactams[a]

	ECS model				Scopolamine model		
drug	dose, mg/kg	no. of animals	median (ir)	AA	no. of animals	median (ir)	AA
Sham		294	300 (204–300)	100	246	300 (174–300)	100
control		498	48 (22–140)	0	387	26 (19–115)	0
Aniracetam	100	14	300 (208–300)*	83			
	10	38	52 (13–154)	0			
	1	27	125 (55–242)	29	35	112 (30–189)	18
Oxiracetam	100	38	131 (38–242)*	57			
	10	32	90 (27–142)	8			
	1	31	84 (24–238)	31	23	135 (48–255)	0
Piracetam	100	13	145 (18–208)	39			
	10	30	55 (13–150)	0			
	1	27	59 (18–166)	0	34	68 (28–210)	25
9A	10	24	130 (22–190)	26			
	1	23	125 (18–224)	37	23	178 (30–300)	47
9B	10	24	94 (49–175)	25			
	1	23	25 (18–69)	0	23	70 (25–168)	44
10	10	12	116 (33–172)	0			
	1	10	249 (108–300)	24	11	88 (57–230)	0
11	10	12	83 (41–185)	55			
	1	24	88 (43–152)**	71	24	93 (31–202)*	49
12	10	11	74 (40–132)	14			
	1	12	86 (29–135)	7	22	122 (36–261)	39
13	10	24	115 (54–290)	17			
	1	35	147 (65–232)	18	36	184 (83–293)***	65
14	10	12	67 (25–159)	0			
	1	34	142 (31–300)	21	48	36 (16–88)	15
15	10	24	125 (19–191)	37			
	1	23	174 (20–300)	37	12	22 (13–58)	0
16	10	9	76 (34–237)	42			
	1	12	60 (47–141)	31	12	18 (12–36)	0
17	10	33	67 (30–182)	24			
	1	12	105 (60–140)	50	11	69 (18–113)	0
18	10	12	17 (16–30)	0			
	1	12	40 (16–169)	10	12	29 (22–40)	21
19	10	12	49 (14–244)	12			
	1	10	18 (12–118)	0	32	84 (27–170)	27
20	10	12	87 (37–164)	0			
	1	12	118 (45–196)	0	19	98 (18–136)	5
21	10	24	150 (69–231)	58			
	1	12	201 (82–243)	51	11	44 (24–106)	0
22	10	12	96 (17–228)	9			
	1	24	137 (50–280)	53	10	91 (46–281)	0
23	10	12	103 (28–277)	9			
	1	24	191 (53–300)	55	10	33 (23–290)	0
24	10	12	113 (52–285)	9			
	1	10	94 (35–210)	0	23	30 (20–67)	0
25	10	24	160 (123–233)*	61			
	1	24	143 (90–203)	46	12	82 (19–247)	7
26	10	11	39 (18–82)	0			
	1	12	27 (23–135)	0	12	73 (16–154)	0
27	10	20	66 (19–218)	0			
	1	18	51 (26–160)	0	12	34 (21–63)	0
28	10	11	28 (18–102)	20			
	1	23	30 (15–161)	7	12	25 (16–68)	12
30	10	24	59 (30–176)	25			
	1	12	110 (69–132)	0	48	36 (19–141)	26

[a] Data for retention trial are reported as median latency (s), interquartile range (ir), and AA value for each group in both ECS- and Scopolamine-induced amnesia models. All substances were tested in equimolar doses of 1, 10, 100 mg/kg of Piracetam. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$.

All compounds proved to be ineffective on gross behavior, motor activity, and neurovegetative system in mice, except compound **20**, which had convulsant effects and caused the loss of the animals' righting reflex at equimolar doses of 100 mg/kg ip of Piracetam.

These experimental results lead to the following considerations:

(1) CI proved to be a good parameter in evaluating the activity of substances, within a screening phase, allowing a meaningful scaling of activities independent of strictly probabilistic costraints. Through the AA values, substances can be easily classified in a rank with relation to their own amnesia-reversal activity. CI's can be utilized as scores for parameterizing the biological activities of molecules including data distribution features. Thus, CI constitutes a good candidate for parameterization of the "biological score" in QSAR procedures, even in instances other than nootropic drugs.

(2) The encouraging data obtained by low-dose treatments with 7- and 5-membered 3-acylamino lactams, as well as their high tolerability, seemingly hold for the validity of our pharmacophore model and for these substances playing a role as a new class of nootropic agents.[25]

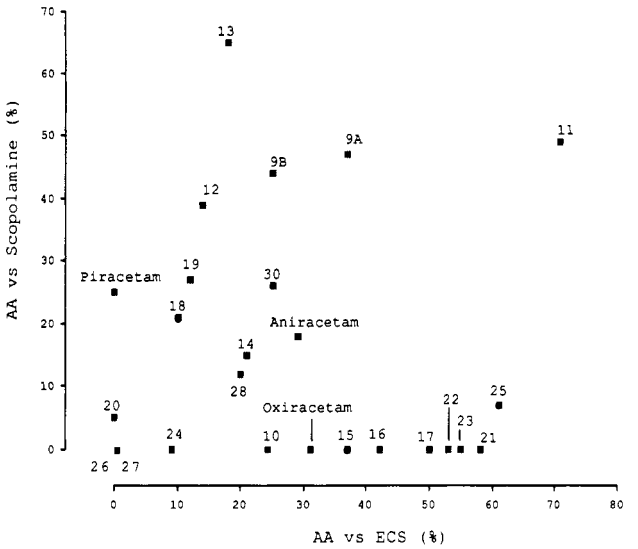


Figure 1. Graphic representation of the amnesia-reversal activity (AA) percent obtained for each substance tested in the ECS- and Scopolamine-induced amnesia models. For the ECS model, the highest scores obtained with either dose levels used are reported.

Experimental Section

Chemistry. Melting points were determined by the capillary method on a electrothermal apparatus (Buchi 535) and are uncorrected. ^{1}H NMR spectra were taken on a Varian VXR 300-MHz FT spectrometer; chemical shifts were expressed in δ values downfield from Me_4Si; the coupling constants are expressed in hertz; exchange with D_2O was used, when necessary, to identify NH protons. The following abbreviations have been used: s, singlet; d, doublet; t, triplet; m, multiplet(s); br, broad. Elemental analyses were performed by an EA 1108 elemental analyzer (Carlo Erba) apparatus and agreed with theoretical values within 0.40%. TLC was carried out with 0.25-mm silica gel 60 F254 (Merck) glass plates. Chromatographic separation was carried out by flash column chromatography using 40–63-mm silica gel (Merck) as stationary phase.

(*S*)-3-Amino-2-pyrrolidinone was prepared as described by Pellegata et al.[26] 3-Amino-ε-caprolactam, in both *R* and *S* forms, was supplied by Aldrich.

(*S*)-3-[(4-Phenylbutanoyl)amino]-ε-caprolactam (20). Method A. CDI (3.89 g, 24 mmol) was added to 4-phenylbutyric acid (3.28 g, 20 mmol) in CH_2Cl_2 (100 mL) and the resulting solution kept under stirring for 30 min at room temperature. Then, (*S*)-3-amino-ε-caprolactam (2.56 g, 20 mmol) was added and the resulting solution kept with stirring for 24 h at room temperature.

The organic phase was washed with 1 N NaOH (3 × 30 mL), 1 N HCl (3 × 30 mL), H_2O, and NaCl saturated solution, dried over anhydrous Na_2SO_4, and filtered. The filtrate was concentrated at reduced pressure to yield 5 g (90%) of **20**: mp 106–108 °C, TLC eluant EtOAc R_f = 0.28; ^{1}H NMR ($CDCl_3$) δ 7.3–7.15 (m, 5 H, aromatics), 6.9 (br d, 1 H, CHN*H*CO), 6.55 (br t, 1 H, CH_2NHCO), 4.5 (m, 1 H, C*H*NCO), 3.35–3.15 (m, 2 H, CH_2NCO), 2.62 (t, J = 7 Hz, 2 H, CH_2Ph), 2.22 (t, J = 7 Hz, 2 H, $NCOCH_2CH_2$) 2.1–1.66 (m, 6 H, $CH_2CH_2CH_2CHNCOCH_2CH_2$), 1.55–1.28 (m, 2 H, $CH_2CH_2CH_2CHNCO$).

(*S*)-3-[[3-(Trifluoromethyl)benzoyl]amino]-ε-caprolactam (13). Compound **13** was prepared according to method A. The crude product was chromatographed (EtOAc/MeOH, 8:2) to give **13** in 74% yield: mp 218–219 °C; TLC eluant EtOAc/MeOH (5:5) R_f = 0.5; ^{1}H NMR ($CDCl_3$/DMSO-d_6) δ 8.15 (s, 1 H, aromatic), 8.05 (d, J = 7 Hz, 1 H, aromatic), 7.9 (br, 1 H, *H*NCOPh), 7.78 (d, J = 7 Hz, 1 H, aromatic), 7.6 (t, J = 7 Hz, 1 H, aromatic), 7.35 (br, 1 H, CH_2NHCO), 4.75–4.65 (m, 1 H, C*H*NCO), 3.40–3.22 (m, 2 H, CH_2NCO), 2.25–1.35 (m, 6 H, $CH_2CH_2CH_2CHN$).

(*R*)-3-[[3-(Trifluoromethyl)benzoyl]amino]-ε-caprolactam (14). Compound **14** was prepared as compound **13** starting from (*R*)-3-amino-ε-caprolactam.

(*S*)-3-[(4-Methoxybenzoyl)amino]-ε-caprolactam (15). Compound **15** was prepared according to method A. Chromatography was carried out on SiO_2 using EtOAc–MeOH (9:1) as eluant. The product thus obtained was taken up with CH_2Cl_2 and precipitated with Et_2O to give **15** in 50% yield: mp 170–172 °C; TLC eluant EtOAc R_f = 0.3; ^{1}H NMR ($CDCl_3$) δ 7.82 (d, J = 10 Hz, 2 H, aromatics), 7.58 (br d, 1 H, CHN*H*CO), 6.93 (d, J = 10 Hz, 2 H, aromatics), 6.58 (br t, 1 H, CH_2NHCO), 4.76–4.66 (m, 1 H, C*H*NCO), 3.85 (s, 3 H, OCH_3), 3.42–3.22 (m, 2 H, CH_2NCO), 2.3–1.35 (m, 6 H, $CH_2CH_2CH_2CHN$).

(*S*)-3-[(3,4,5-Trimethoxybenzoyl)amino]-ε-caprolactam (16). Compound **16** was prepared according to method A, yield 51%: mp 95–97 °C; TLC eluant EtOAc–MeOH (95:5) R_f = 0.28; ^{1}H NMR ($CDCl_3$) δ 7.65 (br d, 1 H, *H*NCOPh), 7.10 (s, 2 H, aromatics), 6.75 (br t, 1 H, CH_2NHCO-), 4.78–4.68 (m, 1 H, C*H*NCO), 3.94 (s, 6 H, 2 *m*-OCH_3), 3.88 (s, 3 H, *p*-OCH_3), 3.42–3.22 (m, 2 H, CH_2NHCO), 2.28–1.35 (m, 6 H, $CH_2CH_2CH_2CHN$).

(*S*)-3-(Formylamino)-ε-caprolactam (11). Compound **11** was prepared according to method A. After 24 h of stirring at room temperature, Et_2O was added until complete precipitation took place; the precipitate was collected by filtration and further purified by crystallization from $CHCl_3/Et_2O$ to give **11** in 48% yield: mp 176 °C; TLC eluant EtOAc R_f = 0.1; ^{1}H NMR (DMSO-d_6) δ 8.25 (br, 1 H, *H*NCHO), 8.1 (s, 1 H, C*H*O), 7.9 (br, 1 H, *H*NCO), 4.6–4.45 (m, 1 H, C*H*NCO), 3.35–3.05 (m, 2 H, CH_2NCO), 2–1.60 (m, 4 H, $CH_2CH_2CH_2CHN$), 1,55–1.20 (m, 2 H, $CH_2CH_2CH_2CHN$).

(*S*)-3-[[(2-Oxopyrrolidin-1-yl)acetyl]amino]-ε-caprolactam (18). Compound **18** was prepared according to method A. After 24 h of stirring, Et_2O was added to the mixture; the precipitate was collected by filtration and further purified by crystallization from CH_3CN to give **18** in 50% yield: mp 245–247 °C; TLC eluant EtOAc/MeOH (7:3) R_f = 0.43; ^{1}H NMR (D_2O) δ 4.65 (d, J = 10 Hz, 1 H, C*H*NHCO), 4.05 (s, 2 H, $COCH_2NCH_2$), 3.5 (t, J = 6 Hz, 2 H, $COCH_2NCH_2$), 3.4–3.2 (m, 2 H, CH_2NHCO), 2.5 (t, J = 8 Hz, 2 H, CH_2NCOCH_2), 2.20–1.30 (m, 8 H, $CH_2CH_2CH_2CHN$, CH_2CH_2CON).

(*S*)-3-[[3-(Trifluoromethyl)benzoyl]amino]-2-pyrrolidinone (21). Compound **21** was prepared according to method A. After workup, the organic phase was evaporated at reduced pressure and the residue crystallized from EtOAc/*n*-hexane to give **21** in 40% yield: mp 133–155 °C; TLC eluant EtOAc/MeOH (9:1) R_f = 0.37; ^{1}H NMR ($CDCl_3$) δ 8.08 (s, 1 H, aromatic), 7.98 (d, J = 7 Hz, 1 H, aromatic), 7.8 (br d, 1 H, CHN*H*CO), 7.7 (d, J = 7 Hz, 1 H, aromatic), 7.5 (t, J = 7 Hz, 1 H, aromatic), 6.7 (s, 1 H, CH_2NHCO), 4.7–4.6 (m, 1 H, C*H*NCO), 3.5–3.38 (m, 2 H, CH_2NCO), 2.82–2.7 (m, 1 H, C*H*HCHN), 2.2–2 (m, 1 H, CH*H*CHN).

(*S*)-3-(Formylamino)-2-pyrrolidinone (22). Compound **22** was prepared according to method A. After 24 h of stirring, Et_2O was added to the mixture until complete precipitation took place; the precipitate was collected by filtration and chromatographed (EtOAc/MeOH, 8:2). The product thus obtained was further purified by treatment with refluxing $CHCl_3$ to give **22** in 44% yield: mp 177–179 °C; TLC eluant EtOAc/MeOH (7:3) R_f = 0.4; ^{1}H NMR (D_2O) δ 8.15–8.05 (2 s, 1 H, NC*H*O), 4.62–4.4 (2 m, 1 H, C*H*NHCO), 3.48–3.3 (m, 2 H, CH_2NCO), 2.6–2.45 (m, 1 H, C*H*HCHN), 2.15–2 (m, 1 H, CH*H*CHN).

(*S*)-3-[[(Benzyloxycarbonyl)-(*S*)-prolyl]amino]-ε-caprolactam (19). Method B. EEDQ (3.45 g, 13.95 mmol) was added with stirring to (*S*)-(benzyloxycarbonyl)proline (2.9 g, 11.63 mmol) in CH_3CN (100 mL) and (*S*)-3-amino-ε-caprolactam (1.49 g, 11.63 mmol) was added after 30 min. The mixture was kept at room temperature with stirring for 24 h, concentrated under vacuum, and chromatographed (EtOAc) to yield 2.9 g (70%) of **19**: mp 156–158 °C; TLC eluant EtOAc R_f = 0.23; ^{1}H NMR ($CDCl_3$) δ 7.5–7.2 (m, 6 H, aromatics, CH_2NHCO), 6.55 (br d, 1 H, CH_2NHCO), 5.25–5.05 (m, 2 H, OCH_2Ph), 4.55–4.20 (m, 2 H, NCOC*H*NCO, C*H*NCOO) 3.7–3.42 (m, 2 H, CH_2NCOO), 3.35–2.15 (m, 2 H, CH_2NHCO), 2.25–1.2 (m, 10 H, $CH_2CH_2CH_2CH$, CH_2CH_2CHN).

(*S*)-3-(Nicotinoylamino)-ε-caprolactam (17). Compound **17** was prepared according to method B. Chromatography was carried out (EtOAc/MeOH, 8:2) to give **17** in 57% yield: mp 177 °C; TLC eluant EtOAc/MeOH (8:2) R_f = 0.38; ^{1}H NMR ($CDCl_3$) δ 9.08 (m, 1 H, aromatic), 8.75 (m, 1 H, aromatic), 8.15 (m, 1 H,

aromatic), 7.75 (br d, 1 H, CHN*H*CO), 7.40 (m, 1 H, aromatic), 6.40 (br t, 1 H, CH_2NHCO), 4.76–4.68 (m, 1 H, C*H*NCO), 3.42–3.22 (m, 2 H, CH_2NCO), 2.30–1.38 (m, 6 H, $CH_2CH_2CH_2CHN$).

(*S*)-3-(Acetylamino)-ε-caprolactam (12). Method C. A solution of acetyl chloride (1.35, 17.17 mmol) in CH_2Cl_2 (10 mL) was added to an ice-cold solution of (*S*)-3-amino-ε-caprolactam (2.2 g, 17.16 mmol) and triethylamine (1.91 g, 18.88 mmol) in CH_2Cl_2 (50 mL). After 24 h of stirring at room temperature, Et_2O was added until complete precipitation took place; the filtrate was evaporated at reduced pressure and the residue chromatographed (EtOAc) to yield 1.8 g (62%) of **12**: mp 147–149 °C; TLC eluant EtOAc/MeOH (8:2) R_f = 0.47; ^{1}H NMR ($CDCl_3$) δ 7.2–7 (br, 2 H, N*H*COCHN*H*CO), 4.6–4.5 (m, 1 H, C*H*NCO), 3.4–3.2 (m, 2 H, CH_2NCO), 2.15–1.75 (m, 7 H, $CH_2CH_2CH_2CHNCOCH_3$), 1.60–1.3 (m, 2 H, CH_2CH_2CHNCO).

(*S*)-1-[(Methoxycarbonyl)methyl]-3-[(4-phenylbutanoyl)amino]-ε-caprolactam (27). NaH 80% in mineral oil (372.82 mg, 10.92 mmol) was added to a solution of **20** (1.5 g, 5.46 mmol) and $BrCH_2COOCH_3$ (1.672 g, 10.92 mmol) in CH_3CN (80 mL) in two aliquots over 1 h. The resulting mixture was kept with stirring at room temperature for 24 h and then filtered. The filtrate, brought to dryness, was chromatographed (EtOAc) to yield 1.4 g (74%) of **27** as an oily product: TLC eluant EtOAc R_f = 0.55; ^{1}H NMR ($CDCl_3$) δ 7.3–7.15 (m, 5 H, aromatics), 6.9 (br d, 1 H, CHN*H*CO), 4.72–4.62 (m, 1 H, C*H*NCO), 4.18 (s, 2 H, NCH_2CO), 3.78–3.65 (m, 4 H, OCH_3, CH*H*NCO), 3.25–3.15 (m, 1 H, C*H*HNCO), 2.65 (t, J = 7 Hz, 2 H, CH_2Ph), 2.2 (t, J = 7 Hz, 2 H, $NCOCH_2CH_2$), 2.1–1.75 (m, 6 H, $CH_2CH_2CH_2CHNCOCH_2CH_2$), 1.7–1.45 (m, 2 H, $CH_2CH_2CH_2CHN$).

(*S*)-1-[(Aminocarbonyl)methyl]-3-[(4-phenylbutanoyl)amino]-ε-caprolactam (26). Gaseous NH_3 was bubbled for 30 min into a solution of **27** (1.6 g, 4.62 mmol) in MeOH (50 mL) cooled with an ice bath. The solution was kept under stirring at room temperature for 24 h, the solvent was evaporated, and the residue was chromatographed (EtOAc/MeOH, 9:1) to yield 1.3 g (85%) of **26**: mp 153–155 °C; TLC eluant EtOAc/MeOH (9:1) R_f = 0.3; ^{1}H NMR ($CDCl_3$) δ 7.31–7.13 (m, 5 H, aromatics), 6.87 (br d, 1 H, CHN*H*CO), 6.42 (br s, 1 H, CON*H*H), 5.89 (br s, 1 H, CONH*H*), 4.76–4.66 (m, 1 H, C*H*NCO), 4.29 (d, J = 15 Hz, 1 H, NC*H*HCON), 3.82 (d, J = 15 Hz, 1 H, NCH*H*CON), 3.76–3.64 (m, 1 H, $CH_2CHHNCO$), 3.35–3.24 (m, 1 H, $CH_2CHHNCO$), 2.63 (t, J = 7 Hz, 2 H, CH_2Ph), 2.2 (t, J = 7 Hz, 2 H, $NCOCH_2CH_2$), 2.08–1.74 (m, 6 H, $CH_2CH_2CH_2CHNCOCH_2CH_2$), 1.58–1.38 (m, 2 H, CH_2CH_2CHN).

(*S*)-1-(2-Hydroxyethyl)-3-[(4-phenylbutanoyl)amino]-ε-caprolactam (28). $NaBH_4$ (402 mg, 10.7 mmol) was added to **27** (1 g, 2.88 mmol) in *tert*-butyl alcohol (18 mL), the temperature was brought to 80 °C, and MeOH (3 mL) was added slowly. The resulting mixture was kept at the reflux temperature for 2 h. After cooling in an ice bath, H_2O was added and the alcohols were evaporated under vacuum; the aqueous phase was extracted with EtOAc, and the solvent was evaporated. The residue was chromatographed (EtOAc/MeOH, 9:1) to yield 530 mg (57%) of **28** as an oily product: TLC eluant EtOAc/MeOH (9:1) R_f = 0.4; ^{1}H NMR ($CDCl_3$) δ 7.32–7.14 (m, 5 H, aromatics), 6.82 (br d, 1 H, CHN*H*CO), 4.7–4.6 (m, 1 H, C*H*NHCO), 3.82–3.48 (m, 5 H, CH*H*NCH_2CH_2OH), 3.38–3.26 (m, 1 H, C*H*HNCH_2CH_2OH), 2.8 (br s, 1 H, O*H*), 2.64 (t, J = 7 Hz, 2 H, CH_2Ph) 2.23 (t, J = 7 Hz, 2 H, $NCOCH_2CH_2$), 2.08–1.76 (m, 6 H, $CH_2CH_2CH_2CHNCOCH_2CH_2$), 1.54–1.36 (m, 2 H, CH_2CH_2CHN).

(*S*)-1-(Formylmethyl)-3-[(4-phenylbutanoyl)amino]-ε-caprolactam (30). A solution of **20** (6 g, 21 mmol) in THF (90 mL) was added dropwise to a suspension of KH (35% in mineral oil, 3.726 g, 32 mmol, washed with pentane) in THF (90 mL). The resulting mixture was kept at reflux temperature until hydrogen development was over. Bromoacetaldehyde dimethyl acetal (7.38 g, 43 mmol) was added dropwise, and the resulting mixture was kept at the reflux temperature overnight. The mixture was evaporated under vacuum, and a saturated NH_4Cl solution (200 mL) was added thereto; the resulting mixture was extracted with CH_2Cl_2 (3 × 70 mL) and the organic phase dried over anhydrous Na_2SO_4.

The residue obtained following solvent evaporation was chromatographed using EtOAc/hexane (95:5) as eluant to obtain 4 g of (*S*)-1-(2,2-dimethoxyethyl)-3-[(phenylbutanoyl)amino]-ε-caprolactam **29** (TLC eluant EtOAc, R_f = 0.5) which was dissolved in CH_3CN (100 mL) and H_2O (100 mL). Trifluoroacetic acid (2 mL) was added, the solution was stirred overnight at room temperature, the CH_3CN was evaporated under vacuum, and the residue was extracted with $CHCl_3$ (3 × 50 mL). The organic phase was washed with a saturated NaCl solution, dehydrated over Na_2SO_4, and evaporated to yield 2.3 g (35% overall) of **30**: TLC eluant EtOAc R_f = 0.31; ^{1}H NMR ($CDCl_3$) δ 9.55 (s, 1 H, C*H*O), 7.30–7.15 (m, 5 H, aromatics), 6.90 (br d, 1 H, N*H*CO), 4.75–4.65 (m, 1 H, C*H*NHCO), 4.25 (m, 2 H, NCH_2CHO), 3.80–3.65 (m, 1 H, C*H*HNCO), 3.15–3.05 (m, 1 H, CH*H*NCO), 2.62 (t, J = 7 Hz, 2 H, CH_2Ph), 2.22 (t, J = 7 Hz, 2 H, $NCOCH_2CH_2$), 2.10–1.75 (m, 6 H, $CH_2CH_2CH_2CHNCOCH_2CH_2$), 1.65–1.45 (m, 2 H, CH_2CH_2CHNCO).

(*S*)-1-[(Methoxycarbonyl)methyl]-3-(formylamino)-ε-caprolactam (24). Compound **24** was prepared as compound **27** (starting from **11** instead of **20**) in 70% yield: mp 60–62 °C; TLC eluant EtOAc R_f = 0.24; ^{1}H NMR ($CDCl_3$) δ 8.15 (s, 1 H, NC*H*O), 7.10 (br, 1 H, N*H*CHO), 4.78–4.68 (m, 1 H, C*H*NCO), 4.18 (s, 2 H, NCH_2CO), 3.78–3.62 (m, 4 H, OCH_3, CH$_2$CH*H*NCO), 3.25–3.15 (m, 1 H, $CH_2CHHNCO$), 2.15–1.48 (m, 6 H, $CH_2CH_2CH_2CHN$).

(*S*)-1-[(Aminocarbonyl)methyl]-3-(formylamino)-ε-caprolactam (23). Compound **23** was prepared as compound **26** (starting from **24** instead of **27**). As chromatography eluant, EtOAc/MeOH (7:3) was used: yield 80%; mp 208–210 °C; TLC eluant EtOAc/MeOH (7:3) R_f = 0.46; ^{1}H NMR (D_2O) δ 8.08 (s, 1 H, NC*H*O), 4.9 (m, 1 H, C*H*NCO), 4.39 (d, J = 16.6 Hz, 1 H, NCH*H*CON), 3.98 (d, J = 16.6 Hz, 1 H, NC*H*HCON), 3.85–3.75 (m, 1 H, $CH_2CHHNCO$), 3.42–3.3 (m, 1 H, $CH_2CHHNCO$), 2.05–1.48 (m, 6 H, $CH_2CH_2CH_2CHN$).

(*S*)-1-(2-Hydroxyethyl)-3-(formylamino)-ε-caprolactam (25). Compound **25** was prepared as compound **28** (starting from **24** instead of **27**). The aqueous phase was evaporated under vacuum and the residue chromatographed (EtOAc/MeOH, 95:5) to give **25** in 55% yield: mp 89–92 °C; TLC eluant EtOAc/MeOH (95:5) R_f = 0.33; ^{1}H NMR ($CDCl_3$) δ 8,1 (s, 1 H, NC*H*O), 7.1 (br s, 1 H, N*H*CHO), 4.8–4.65 (m, 1 H, C*H*NCO), 3.9–3.45 (m, 5 H, C*H*HNCH_2CH_2OH), 3.4–3.26 (m, 1 H, CH*H*NCH_2CH_2OH), 2.85 (br s, 1 H, O*H*), 2.14–1.76 (m, 4 H, $CH_2CH_2CH_2CHN$), 1.58–1.36 (m, 2 H, $CH_2CH_2CH_2CHN$).

Molecular Modeling. Conformational analysis of candidate compounds was carried out using the MM2[11] force field included in the molecular modeling software MACROMODEL,[27] running on a Personal IRIS 4D/25 workstation. The MM2 force field was selected on the basis of our previous study:[28] a comparison made with ab initio calculations showed that it was more reliable, in the study of conformational properties of compounds containing a pyrrolidinonic ring, than the AMBER[29] and OPLS[30] force fields and the quantum mechanical semiempirical AM1 method.[31]

For each compound, conformational analysis was performed generating conformational isomers by the MULTICONF[32] option of MACROMODEL: ring conformational isomers were generated choosing a breaking bond in the ring and systematically increasing the ring torsions by 30° in the 0–360° range; all the structures presenting values of the ring closure distance, i.e. of the broken bond, within a predefined range (1.0–2.0 Å) were retained. Relevant torsional angles of the side chains were increased by 30° while amidic bonds were increased by 180°. The resulting structures were completely optimized by the Block Diagonal Newton Raphson procedure using as convergence criteria a gradient variation lower than 0.01 kcal mol^{-1} Å^{-1}; the nature of the stationary points was tested by computing eigenvalues of the second-derivative matrix.

Pharmacology. (A) Amnesia-Reversal Testing. (1) Experimental Procedure. Experiments were carried out on male CD1 mice (Charles River Italia), weighing 24–28 g. The animals were housed in transparent makrolon cages and received tap water and standard laboratory diet (4RF21, Mucedola S.r.l. Italia) ad libitum. The animal rooms were maintained under standard conditions of temperature (22 ± 1 °C) and relative humidity (55 ± 10%), with a 12:12-h light/dark cycle, and 12–15 filtered air changes/h.

The method used was similar to the one described by Ader et al.[33] envisaging acquisition followed by retention trials. The apparatus for the passive avoidance test consisted of a dark plastic

box (42 × 42 × 40 cm high), provided with a stainless steel grid floor. Against the front wall was leaned a smaller white chamber (30 × 10 × 12 cm high), connected to the box through a sliding door (6 × 6 × 6 cm high). A 60-W lamp was fixed 40 cm above the center of the white chamber, while the box was kept dark.

For the acquisition trial, each mouse was placed for 1 min in the light compartment and then allowed to enter the dark box, recording the time it did so in seconds. Mice whose latency was greater than 60 s were discarded. Once the mouse entered the dark box with all four paws, the sliding door was closed, and 3 s afterward a scramblered electric foot-shock (0.21 mA for 2 s) was delivered through the grid floor. The animal was then returned to its home cage until the retention trial, which was carried out at 24 h after the acquisition trial: each mouse was placed back into the light chamber and the time taken to enter the dark box was recorded. End point of retention latency was set at 300 s.

In the passive avoidance test with ECS- or Scopolamine-induced amnesia, the animals were distributed into three groups as follows:

(1) Sham group, to measure the normal retention in the passive avoidance paradigm. The animals were injected vehicle (ip) at 30 min and at 30 and 15 min before the acquisition trial in the ECS- and in the Scopolamine-induced amnesia test, respectively.

(2) Control group, to measure amnesia caused by either ECS or Scopolamine. The animals received ECS (delivered through auricular electrodes; rectangular positive pulses: current = 20 mA, pulse width = 0.6 ms, shock duration = 0.5 s, frequency = 50 Hz) immediately after the acquisition trial, or Scopolamine hydrobromide (1.5 mg/kg sc; Merck) 15 min before the acquisition trial. In both amnesic procedures, each mouse was injected vehicle (ip) 30 min before the acquisition trial.

(3) Treated group, to measure AA of each compound as compared with the amnesic treatment. The animals received either ECS or Scopolamine as described for the control group. The substances were administered 30 min before the acquisition trial.

For each substance, two doses (equimolar to 10 and 1 mg/kg ip of Piracetam) and one dose (equimolar to 1 mg/kg ip of Piracetam) were tested for ECS- and Scopolamine-induced amnesia, respectively. The reference compounds, i.e. Aniracetam, Oxiracetam, and Piracetam, were also tested at the equimolar dose of 100 mg/kg ip of Piracetam in the ECS model. Saline (NaCl, 0.9%) was the vehicle used for water-soluble substances; otherwise, it was a solution of dimethyl sulfoxide–2% Tween 80 (Merck), 1:4 (v:v).

(2) Statistics. Data were reported as median retention latency and interquartile range per group. For each experiment the amnesic effect of Scopolamine or ECS was verified by comparing control groups with Sham groups through the Mann-Whitney's U test. When no significant differences were found ($p > 0.05$), the experiment was discarded. For the accepted experiments, the Mann-Whitney's U test was used to compare the retention latency of treated and control animals. All probabilities were two-tailed.

Results for each compound were also expressed as percent of AA to enable comparison of the activity of each substance. AA is described as follows:

$$AA = (CI_T/CI_S) \times 100$$

where CI_T = confrontation index for the treated group and CI_S = confrontation index for Sham group.

Confrontation Index. This new parameter was introduced for the evaluation of data. The CI is calculated as follows:

$$\frac{U' - U}{n_i n_j} = CI$$

with $U = n_i n_j + n_i(n_i + 1)/2 - R_I$, $U' = n_i n_j - U$, n_i = number of elements of group I (Sham or treated), n_j = number of elements of group J (control), R_I = sum of the ranks assigned to the group I. Throughout this formula, that is a function of the Mann-Whitney's U parameters,[34] it is possible to obtain a descriptive index (confrontation index) which permits a common scale of activities to be made among substances, independently of the number of tested animals. In other words, through the CI, the degree of discrimination between two experimental groups as measured by U statistics can be easily converted into a numerical value which can be inserted in a score scale.

The CI reaches a maximum when $U' = 0$ (CI = 1), corresponding to the total separation of the two experimental groups, and a minimum when $U = U'$ (CI = 0), i.e., complete overlapping.

(B) Observational Tests for CNS Signs. Male CD1 mice (Charles River Italia), weighing 22–24 g, were used and housed under standard conditions as described in A. Compounds were administered as reported in A, at 4 equimolar doses up to 100 mg/kg ip of Piracetam, each to 10-mice groups, whereas the respective control groups were treated with vehicle alone. All the animals were food-deprived starting 18 h before testing. Each test was performed using different animals.

(1) Irwin Test.[35] From drug administration until the successive 6 h, each animal was rated by an expert observer, unaware of the treatment, for the presence or absence of the signs and symptoms of alteration of normal behavioral, neurological, and autonomic conditions, such as changes in motor activity, mood, CNS excitation, motor coordination, muscle tone, reflexes, effects on eyes, secretions/excretions, etc.

(2) Hot-Plate Test.[36] The animals from each dose group were placed on a metal plate, at constant 56 °C, at 30, 60, 120, and 180 min after administration of the compounds. The increased latency (in seconds) of reaction (jumping or licking paws) in comparison with the basal and/or control groups was considered a measure of the analgesic effect. Unpaired two-tailed Student's t test was used as statistical inference for comparison between treated and control groups.

(C) Evaluation of in Vitro Acetyl Cholinesterase (AChE) Inhibition. One male Fischer 344 rat (Charles River Italia) brain was homogenized in 15 mL of Sörensen 66.7 mM, pH 7.2, by addition of a 1% solution of Triton x-100 (2 mL/1 mL of homogenate). The supernatant was used as AChE source. Substances were suspended in H_2O or DMSO and diluted in Sörensen buffer, pH 7.2, dissolved at final concentrations ranging from 10^{-3} to 10^{-11} M. AChE activity was determined as described by Ellman et al.[37] at 37 °C. A Test-Combination Cholinesterase (Boehringer Mannheim Gmgh) kit was used and suited to an autoanalyzer (Cobas Mira S, Roche). Physostigmine was used as reference inhibitor. For this known AChE agonist the concentration causing 50% inhibition (IC_{50}) was about 1.15×10^{-8} M.

Acknowledgment. The authors wish to thank Dr. Paola Piovesan and Mr. Gianni Quatrini for providing Irwin and hot-plate test data and Mr. Mario Vertechy and Ms. Cristina Ciogli for providing data on evaluation of in vitro acetyl cholinesterase inhibition and acknowledge Dr. Sandra Muck and her staff for analytic support. They are also grateful to Mrs. Luisella Mattace for her patient linguistic and editorial assistance.

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