Structure−Antigastrin Activity Relationships of New Spiroglumide Amido Acid Derivatives

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A series of new spiroglumide amido acid derivatives was synthesized and evaluated for their ability to inhibit the binding of cholecystokinin (CCK) to guinea pig brain cortex (CCK_B receptors) and peripheral rat pancreatic acini (CCK_A receptors), as well as to inhibit in vitro the gastrin-induced Ca^{2+} increase in rabbit gastric parietal cells. Appropriate chemical manipulations of the structure of spiroglumide (CR 2194), i.e., (*R*)-4-(3,5-dichlorobenzamido)-5-(8-azaspiro[4.5]decan-8-yl)-5-oxopentanoic acid, led to potent and selective antagonists of CCK_B/gastrin receptors. Structure−activity relationships are discussed. Some of these new derivatives, as, for example, compound **54** (CR 2622), i.e., (*S*)-4-[[(*R*)-4′-[(3,5-dichlorobenzoyl)-amino]-5′-(8-azaspiro[4.5]decan-8-yl)-5′-oxo-pentanoyl]amino]-5-(1-naphthylamino)-5-oxopentanoic acid, exhibit activity 70−170 times greater than that of spiroglumide, depending upon the model used ($IC_{50} = 2 \times 10^{-8}$ vs 140×10^{-8} mol in binding inhibition of [^{3}H]-N-Me-N-Leu-CCK-8 in guinea pig brain cortex and $IC_{50} = 0.7 \times 10^{-8}$ vs 122.3×10^{-8} mol in inhibition of gastrin-induced Ca^{2+} mobilization in parietal cells of rabbit, respectively). Computer-assisted conformational analysis studies were carried out in order to compare the chemical structure of both the agonist (pentagastrin) and the antagonist (**54**).

The gastrointestinal polypeptide hormones gastrin and cholecystokinin (CCK) are chemically closely related. Both have a common terminal pentapeptide sequence, but they exhibit different biological effects on their target tissues.[1,2] CCK is also widely distributed throughout the brain, and it has been hypothesized that it may function as a neurotransmitter in the central nervous system (CNS).[3]

The peripheral actions of CCK are mediated by a receptor subtype termed CCK_A, while the central actions are mainly mediated by the subtype receptor termed CCK_B, for which the minimum agonist ligand requirement is tetragastrin (CCK-4).[4] A third receptor subtype, which appears to be closely related to the CCK_B type, is the stomach gastrin receptor.

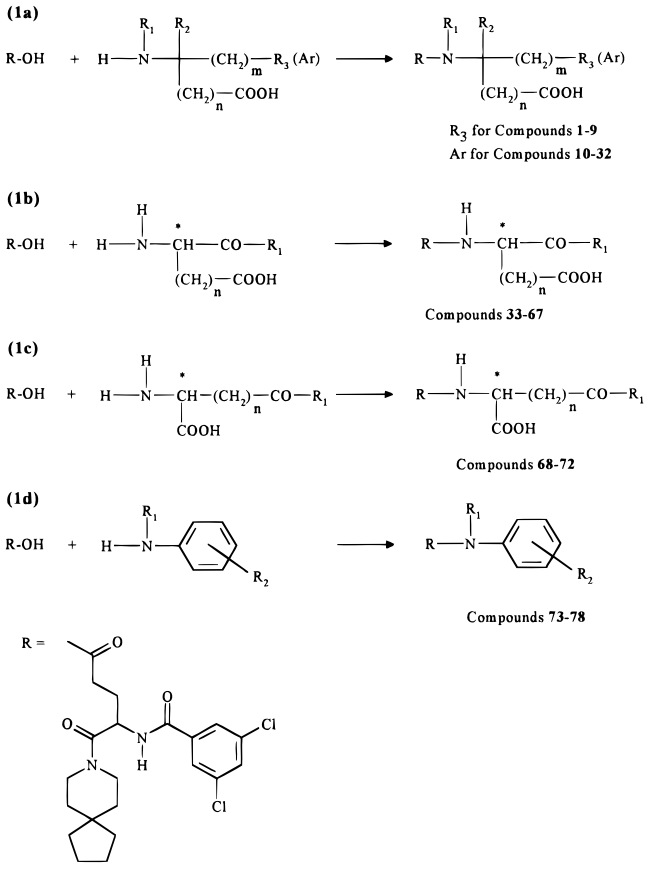
Recently, several potent and specific CCK_B antagonists have been discovered, such as compound L-365,-260[6] or the α-methyltryptophan derivative CI-988.[7] Spiroglumide (CR 2194), i.e., (*R*)-4-(3,5-dichlorobenzamido)-5-(8-azaspiro[4.5]decan-8-yl)-5-oxopentanoic acid, is a competitive and specific CCK_B antagonist, with affinity for the CCK_B receptor ($K_i \sim 10^{-7}$M).[8] Its antigastrin activity was demonstrated in vivo on several animal species and models, even after oral administration.[9]

The aim of our investigation was to explore the possibility that appropriate chemical manipulations of the structure of spiroglumide could lead to new molecular entities exhibiting high affinity (nanomolar range) and selective antagonism on CCK_B/gastrin receptors.

Chemistry

For the synthesis of compounds **1**−**78**, we have utilized the mixed anhydride method[10] illustrated by Scheme 1. Therefore spiroglumide was reacted in THF at −10 °C with triethylamine and ethyl chloroformate for about 15−20 min. The appropriate amino acids of the formulae shown in Scheme 1 were dropped in the reaction mixture dissolved in aqueous solution at the same temperature, and the reaction continued for 1 h at −10 °C and for 12 h at room temperature to give the

Scheme 1. Synthesis of Compounds **1**−**78** Summarized in Tables 1−5[a]



[a] Reagents: Et_3N, EtOCOCl, −10 °C.

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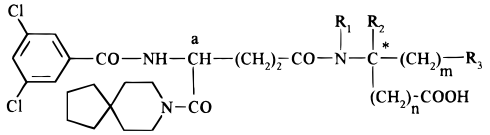
Table 1. Physical and Biological Properties of Spiroglumide Amides with Aliphatic Amino Acids



compd	R_1	R_2	m	n	R_3	conf (*)	mp, °C	recryst solvent	optical[b] rotation, deg	formula	IC_{50}, 10^{-8} mol: CCK_B (central)[c]	IC_{50}, 10^{-8} mol: gastrin (peripheral)[d]
1	H	H	0	0	H		86	AcOEt/iPr_2O, 1:4	−39.1	$C_{23}H_{29}Cl_2N_3O_5$	101.7 (92.0−113)	104.3 (53.3−204)
2	CH_3	H	0	0	H		104	AcOEt/iPr_2O, 2:3	−39.6	$C_{24}H_{31}Cl_2N_3O_5$	156.6 (82.6−298)	281 (129−611)
3	H	H	0	1	H		73	AcOEt/iPr_2O, 1:4	−40	$C_{24}H_{31}Cl_2N_3O_5$	71.3 (39.4−129)	48.8 (20.3−117)
4	H	H	0	2	H		95	AcOEt/iPr_2O, 1:3	−42.3	$C_{25}H_{33}Cl_2N_3O_5$	119.5 (94.0−152)	60.0 (18.1−199)
5	H	CH_3	0	0	H	*RS*	111	AcOEt/iPr_2O, 1:2	−36.6	$C_{24}H_{31}Cl_2N_3O_5$	125.2 (108−145)	99.4 (34.7−285)
6	H	H	3	0	methyl	*RS*	77	iPr_2O	−32.5	$C_{27}H_{37}Cl_2N_3O_5$	105.6 (80.6−138)	9.5 (5.3−17.1)
7	H	H	1	0	1-cyclohexyl	*R*	155	AcOEt/iPr_2O, 1:4	−23.9	$C_{30}H_{41}Cl_2N_3O_6$	61.2 (30.4−123)	14.2 (8.1−24.9)
8	H	H	2	0	thiomethyl	*RS*	104	AcOEt/iPr_2O, 2:5	−11.1[e]	$C_{26}H_{35}Cl_2N_3O_5S$	53.9 (46.1−63.1)	31.6 (13.4−74.7)
9	H	H	1	0	isopropyl	*RS*	86	AcOEt/iPr_2O, 1:3	−31.2	$C_{27}H_{37}Cl_2N_3O_5$	35.5 (17.0−74.1)	5.1 (2.0−12.8)
spiroglumide											140.0 (98.9−198)	122.3 (31.3−477)

[a] Rectus configuration. [b] $[\alpha]_D$ in $CHCl_3$ ($c = 2$). [c] IC_{50}: 10^{-8} mol displacing concentration and $p = 0.05$ fiducial limits required to inhibit by 50% the specific binding of 0.45 nmol of [^{3}H]-N-Me-N-Leu-CCK-8 in guinea pig brain cortex (CCK_B). [d] IC_{50}: 10^{-8} mol concentration and $p = 0.05$ fiducial limits required to inhibit by 50% the gastrin-induced [Ca^{2+}] cytosolic elevation. [e] $[\alpha]_D$ in MeOH ($c = 2$).

resultant acidic amido derivatives of spiroglumide (**1**−**78**). The physicochemical characteristics of these new compounds are given in Tables 1−5.

Results and Discussion

The results obtained from binding and gastrin antagonism are presented in Tables 1−5. Biological activity was assessed by evaluating the CCK_B antagonism on guinea pig brain cortex binding studies and by studying increases in cytosolic Ca^{2+} induced by gastrin in isolated rabbit parietal cells.

Initially, we synthesized a series of amides of spiroglumide with aliphatic amino acids (compounds **1**−**9**). Among these amides, the derivative of spiroglumide with DL-leucine (compound **9**) displayed the highest activity on both models, 35.5×10^{-8} and 5.1×10^{-8} M, respectively; i.e., this compound is on an average about 10 times more potent than the parent compound spiroglumide (Table 1).

Because of this encouraging result, a series of spiroglumide amides with aromatic amino acids was synthesized. The results obtained with these derivatives (compounds **10**−**32**) from binding and gastrin antagonism are presented in Table 2. The derivative of spiroglumide with DL-phenylalanine (compound **10**) exhibited a potency about 10−20 times higher than that of spiroglumide on both the central and peripheral CCK_B/gastrin models. The corresponding *R*,*R*-diastereoisomer (compound **12**) of the racemic **10** was about 5 times more potent than the corresponding *R*,*S*-diastereoisomer **13**. The introduction in R_1 of **12** of a methyl group (compound **15**) does not change the CCK_B/gastrin antagonism activity. It is interesting to note that compound **11**, the DL-phenylalanine amide of (*S*)-spiroglumide, is completely devoid of any anti-CCK_B/gastrin activity.

The introduction of substituents, such as 4-hydroxy or 4-chloro, in the phenyl ring of phenylalanine (compounds **16** and **17**, respectively) is less favorable. The same happens with the introduction of a methyl group in R_2 (compound **19**), as well as by increasing or shortening the distance between the chiral center (*) and the aromatic ring of the phenylalanine moiety (compound **20**, $m = 0$, and compound **21**, $m = 2$, respectively). The increase in the length of the alkylcarboxylic chain ($n = 1$ or 2, compounds **22**−**24**), as well as the substitution of the phenyl moiety of phenylalanine with the 2-pyridyl ring (compound **18**), does not seem to increase the affinity for the CCK_B/gastrin receptor.

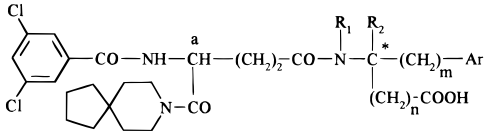
On the contrary, the introduction in Ar of the 3-indolyl group produced compounds with high CCK_B/gastrin receptor affinity (compounds **25**−**30**). Also in this case, the best results were obtained with the *R*,*R*-derivative **26**, which is on an average about 50 times more potent than the parent compound spiroglumide. Compound **26** is about 3−5 times more potent than its corresponding *R*,*S*-diastereoisomer **27**.

The introduction of an *N*-methyl group in R_1 (compound **28**) does not significantly change the CCK_B/gastrin antagonism activity. The same happens by introducing a methylene group between the chiral center (*) and the carboxylic acid ($n = 1$, compound **30**). Also the introduction in Ar of the 1-naphthyl group gave good results (compound **31**, $IC_{50} = 6.7 \times 10^{-8}$ and 0.9×10^{-8} M on the brain binding and parietal cells, respectively), whereas the introduction of a 2-naphthyl group (compound **32**) was less favorable.

The amidation of spiroglumide with α-amidoglutamic and -aspartic acid derivatives led to several compounds exhibiting potent CCK_B/gastrin receptor antagonism (Table 3). When R_1 is the 8-azaspiro[4.5]decan-8-yl substituent and *n* is 2, the corresponding compounds **33** and **34** (*R*,*R*- and *R*,*S*-diastereoisomers, respectively) exhibit different CCK_B/gastrin affinity because their activity depends on the configuration of the chiral center (*). Compound **33**, the *R*,*S*-diastereoisomer, is indeed about 20 times more effective than its *R*,*R*-analogue **34**, which has, on average, the same activity as its corresponding aspartic acid derivative **35** ($n = 1$). Besides, it is noteworthy that **33** is about 20 times more effective than spiroglumide in displacing radioligand from CCK_B receptors and inhibiting gastrin receptor function.

The introduction in R_1 of other aliphatic amino groups is less favorable (compounds **36**−**42**), with the exception

Table 2. Physical and Biological Properties of Spiroglumide Amides with Aromatic Amino Acids



compd	R_1	R_2	*m*	*n*	Ar	conf (*)	mp, °C	recryst solvent	optical[b] rotation, deg	formula	IC_{50}, 10^{-8} mol: CCK_B (central)[c]	IC_{50}, 10^{-8} mol: gastrin (peripheral)[d]
10	H	H	1	0	phenyl	*RS*	109	EtOH/H_2O, 1:1	−47.3	$C_{30}H_{35}Cl_2N_3O_5$	21.9 (16.0−29.9)	5.5 (4.0−7.5)
11[e]	H	H	1	0	phenyl	*RS*	118	EtOH/H_2O, 1:1	+45.3	$C_{30}H_{35}Cl_2N_3O_5$	In (>100)	In (>100)
12	H	H	1	0	phenyl	*R*	109	EtOH/H_2O, 1:1	−51.3	$C_{30}H_{35}Cl_2N_3O_5$	14.4 (10.1−20.8)	3.6 (1.3−9.7)
13	H	H	1	0	phenyl	*S*	118	EtOH/H_2O, 1:1	−36.6	$C_{30}H_{35}Cl_2N_3O_5$	70.7 (42.0−119)	24.1 (13.0−44.7)
14	CH_3	H	1	0	phenyl	*RS*	109	AcOEt/iPr_2O, 1:4	−23.2	$C_{31}H_{37}Cl_2N_3O_5$	21.2 (12.8−35.0)	7.1 (5.0−9.9)
15	CH_3	H	1	0	phenyl	*R*	120	AcOEt/iPr_2O, 1:4	+1.8	$C_{31}H_{37}Cl_2N_3O_5$	10.1 (5.5−18.7)	8.2 (3.4−19.9)
16	H	H	1	0	4-OH-phenyl	*RS*	150	EtOH/H_2O, 1:1	−32.3	$C_{30}H_{35}Cl_2N_3O_6$	32.0 (22.7−45.1)	17.3 (4.5−66.4)
17	H	H	1	0	4-Cl-phenyl	*RS*	119	EtOH/H_2O, 1:1	−37.0	$C_{30}H_{34}Cl_3N_3O_5$	65.9 (53.4−81.3)	16.2 (9.9−26.4)
18	H	H	1	0	2-pyridyl	*R*	103	AcOEt/iPr_2O, 1:3	−73.1	$C_{29}H_{34}Cl_2N_4O_5$	15.5 (7.8−30.5)	30.7 (3.8−247)
19	H	CH_3	1	0	phenyl	*RS*	125	AcOEt/iPr_2O, 1:5	−7.5	$C_{31}H_{37}Cl_2N_3O_5$	79.9 (73.7−86.6)	7.5 (3.5−16.2)
20	H	H	0	0	phenyl	*RS*	107	EtOH/H_2O, 1:1	−27.2	$C_{29}H_{33}Cl_2N_3O_5$	39.0 (27.8−54.7)	8.0 (4.5−14.4)
21	H	H	2	0	phenyl	*RS*	104	AcOEt/iPr_2O, 1:3	−21.6	$C_{31}H_{37}Cl_2N_3O_5$	29.5 (18.9−46.0)	12.6 (4.5−35.3)
22	H	H	1	1	phenyl	*R*	85	amorphous solid	−51.4	$C_{31}H_{37}Cl_2N_3O_5$	21.4 (15.3−29.9)	3.1 (1.8−5.4)
23	H	H	1	1	phenyl	*S*	79	amorphous solid	−37.3	$C_{31}H_{37}Cl_2N_3O_5$	34.3 (23.5−50.1)	19.3 (9.7−38.1)
24	H	H	1	2	phenyl	*RS*	118	AcOEt/iPr_2O, 1:3	−11.0	$C_{32}H_{39}Cl_2N_3O_5$	15.7 (8.3−29.7)	8.6 (4.9−15.2)
25	H	H	1	0	3-indolyl	*RS*	105	AcOEt/iPr_2O, 1:3	−38.4	$C_{32}H_{36}Cl_2N_4O_5$	4.9 (2.4−9.9)	1.2 (0.5−2.9)
26	H	H	1	0	3-indolyl	*R*	139	EtOH/H_2O, 1:2	−29.4	$C_{32}H_{36}Cl_2N_4O_5$	4.0 (2.8−5.9)	0.9 (0.6−1.3)
27	H	H	1	0	3-indolyl	*S*	105	EtOH/H_2O, 1:2	−45.1	$C_{32}H_{36}Cl_2N_4O_5$	21.6 (13.3−35.0)	3.5 (2.3−5.2)
28	CH_3	H	1	0	3-indolyl	*R*	158	AcOEt/iPr_2O, 1:4	−7.4	$C_{33}H_{38}Cl_2N_4O_5$	5.7 (3.1−10.5)	0.7 (0.5−1.0)
29	CH_3	H	1	0	3-indolyl	*S*	111	AcOEt/iPr_2O, 1:4	−45.8	$C_{33}H_{38}Cl_2N_4O_5$	35.2 (21.1−58.6)	5.1 (1.8−14.7)
30	H	H	1	1	3-indolyl	*RS*	121	AcOEt/iPr_2O, 1:3	−20	$C_{33}H_{38}Cl_2N_4O_5$	4.5 (1.8−11.2)	1.3 (0.7−2.3)
31	H	H	1	0	1-naphthyl	*R*	108	EtOH/H_2O, 1:1	−3.6	$C_{34}H_{37}Cl_2N_3O_5$	6.7 (4.6−9.7)	0.9 (0.6−1.4)
32	H	H	1	0	2-naphthyl	*R*	109	AcOEt/iPr_2O, 1:3	−55.5	$C_{34}H_{37}Cl_2N_3O_5$	12.9 (11.0−15.0)	2.4 (1.0−6.1)

[a] Rectus configuration. [b] $[\alpha]_D$ in $CHCl_3$ ($c = 2$). [c,d] See corresponding footnotes to Table 1. [e] *S*-Isomer of spiroglumide.

of compound **37**, in which R_1 is the dipentylamino group, that exhibits about the same activity as **33**. Compound **43**, the decahydroisoquinolin-2-yl analogue of **33**, showed the same activity, whereas the corresponding secondary decahydronaphthalen-2-ylamido derivative **44** is less effective.

The introduction in R_1 of aromatic amino groups, such as *N*-(methylbenzyl)amino and naphth-1-ylamino, gave the best contribution to CCK_B/gastrin receptor affinity. Indeed, as, for example, the α-amidoaspartic acid derivatives in which R_1 is the *N*-(methylbenzyl)amino group, in the *R*,*S*-configuration (compound **48**) or *R*,*R*-configuration (compound **49**), both exhibit an anti-CCK_B/gastrin activity about 40 times greater than that of spiroglumide.

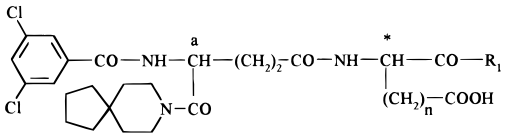
The (naphth-1-ylamino)glutamic acid derivative, having the *R*,*S*-configuration (compound **54**, coded CR 2622) (Figure 1), is the most active compound of this series, displaying an IC_{50} of 2.0×10^{-8} M in the brain binding model and an IC_{50} of 0.7×10^{-8} M in gastric cells. Its anti-CCK_B/gastrin activity is therefore 70 and 170 times higher than that exhibited by spiroglumide, whereas its diastereoisomer (*R*,*R*)-**55** is about 4 times less active. The corresponding aspartic acid derivative **56** ($n = 1$) and the (*R*,*R*)-naphth-2-ylamino derivative **57** are both much less effective, especially on the binding model. Also the introduction in R_1 of substituents such as *N*-methylanilino (compound **50**), phenethylamino (compounds **51** and **52**), and (3-phenyl-1-propyl)amino (compound **53**) produced compounds with scarce affinity for CCK_B/gastrin receptor, if compared with that exhibited by **54**.

The N-methylation of the naphth-1-ylamino group of **54** decreased the activity of the latter compound **58** ($IC_{50} = 6.2 \times 10^{-8}$ and 1.3×10^{-8} M on the brain binding and parietal cells, respectively). Also the introduction of an aromatic nitrogen atom in the quinoline analogue of **54** decreased the CCK_B/gastrin receptor affinity (compounds **59** and **60**). The elongation of the chain between the α-amido group of the glutamic moiety and the naphthyl group slightly decreased the activity (compound **61**). Other attempts for increasing the potency of **54** by saturation of one of two rings of the naphthyl moiety of **54** were, more or less, all unfavorable (compounds **62**−**64**). Also, the introduction in R_1 of tertiary bicyclic (C_9 size) amides (compounds **65**−**67**) produced compounds 5−10 times less effective than **54**.

The amidation of spiroglumide with γ(or β, respectively)-amidoglutamic and -aspartic acid derivatives led to compounds exhibiting potent CCK_B/gastrin receptor antagonism (Table 4, compounds **68**−**72**). The most effective representative of the series is the compound carrying in R_1 the naphth-1-ylamino group; when *n* is 2, the corresponding **72**, which is the γ-isomer of **54**, exhibits a CCK_B/gastrin receptor affinity that is about one-half of that of the latter, whereas the corresponding derivative with $n = 1$ (compound **70**) is about as potent as **72**. Also in this case, the benzylamino and *N*-(methylbenzyl)amino derivatives **68**, **69**, and **71** are less potent than the corresponding naphth-1-ylamino derivatives.

The amidation of spiroglumide with aminobenzoic and aminophenylacetic acids gave derivatives (compounds **73**−**78**) with CCK_B/gastrin receptor affinity depending on the position of the aromatic free carboxylic group (Table 5). The most potent derivative of this series is compound **77** that exhibits a potency about 15 times greater than that of spiroglumide ($IC_{50} = 14.6 \times 10^{-8}$

Table 3. Physical and Biological Properties of Spiroglumide α-Amides of Glutamic or Aspartic Acid Derivatives



compd	R_1	*n*	conf (*)	mp, °C	recryst solvent	optical[b] rotation, deg	formula	IC_{50}, 10^{-8} mol: CCK_B (central)[c]	IC_{50}, 10^{-8} mol: gastrin (peripheral)[d]
33	8-azaspiro[4.5]decan-8-yl	2	*S*	221	MeCN	−4.1[e]	$C_{35}H_{48}Cl_2N_4O_6$	14.1 (12.6−15.8)	3.7 (1.1−12.2)
34	8-azaspiro[4.5]decan-8-yl	2	*R*	134	MeCN	−27.9	$C_{35}H_{48}Cl_2N_4O_6$	169.2 (61.9−462)	20.6 (9.2−46.3)
35	8-azaspiro[4.5]decan-8-yl	1	*R*	117	MeCN/iPr_2O	−16.6	$C_{34}H_{46}Cl_2N_4O_6$	135.1 (51.6−354)	20.0 (11.0−36.3)
36	dipropylamino	2	*S*	154	MeCN	−29.4[f]	$C_{32}H_{46}Cl_2N_4O_6$	29.9 (28.6−31.3)	11.9 (8.1−17.6)
37	dipentylamino	2	*S*	170	95% EtOH	−23.5	$C_{36}H_{54}Cl_2N_4O_6$	13.5 (7.3−25.2)	4.8 (2.8−8.5)
38	(3,3-dimethylbutyl)amino	2	*S*	229	iPrOH	−22.6	$C_{32}H_{46}Cl_2N_4O_6$	26.8 (14.9−48)	23.3 (13.6−39.8)
39	(4,4-dimethylcyclohexyl)amino	2	*S*	230	MeCN/MeOH, 6:1	−43.9	$C_{34}H_{48}Cl_2N_4O_6$	69.1 (35.1−136)	7.0 (5.0−9.7)
40	(4,4-dimethylcyclohexyl)amino	2	*R*	184	MeCN	−15.2	$C_{34}H_{48}Cl_2N_4O_6$	121.9 (89.2−166.6)	11.4 (2.7−48.4)
41	2-adamantylamino	2	*S*	253	DMF/H_2O, 1:1	−19.8[e]	$C_{36}H_{48}Cl_2N_4O_6$	In (>300)	In
42	[2-(1-adamantyl)ethyl]amino	2	*R*	142	MeCN	−10.3	$C_{38}H_{52}Cl_2N_4O_6$	In (>300)	In
43	decahydroisoquinolin-2-yl	2	*S*	176	MeCN	−2.0[e]	$C_{35}H_{48}Cl_2N_4O_6$	18.5 (8.7−39.4)	2.8 (1.1−6.7)
44	decahydronaphthalen-2-ylamino	2	*S*	214	MeCN	−21.2	$C_{36}H_{50}Cl_2N_4O_6$	43.4 (19.9−94.6)	8.9 (3.6−22.1)
45	benzylamino	2	*R*	176	MeCN	−2.5[f]	$C_{33}H_{40}Cl_2N_4O_6$	42.7 (35.3−51.7)	3.8 (1.3−11.2)
46	*N*-(methylbenzyl)amino	2	*R*	121	MeCN	−11.6	$C_{34}H_{42}Cl_2N_4O_6$	25.3 (19.0−33.7)	3.7 (1.9−7.2)
47	*N*-(methylbenzyl)amino	2	*S*	129	MeCN	−11.7	$C_{34}H_{42}Cl_2N_4O_6$	10.1 (8.2−12.4)	2.4 (1.4−4.1)
48	*N*-(methylbenzyl)amino	1	*S*	194	iPrOH	−60.8[e]	$C_{33}H_{40}Cl_2N_4O_6$	3.9 (2.8−5.5)	2.7 (1.2−5.8)
49	*N*-(methylbenzyl)amino	1	*R*	196	MeCN	+85.9[e]	$C_{33}H_{40}Cl_2N_4O_6$	4.9 (2.1−11.3)	0.9 (0.4−1.9)
50	*N*-methylanilino	2	*R*	110	MeCN	−57.6	$C_{33}H_{40}Cl_2N_4O_6$	67.3 (37.6−120.4)	16.6 (5.0−54.5)
51	phenethylamino	2	*R*	128	MeCN	−13.5	$C_{34}H_{42}Cl_2N_4O_6$	110.6 (37.8−324)	39.8 (8.0−198)
52	phenethylamino	1	*S*	116	MeCN	−8.5[e]	$C_{33}H_{40}Cl_2N_4O_6$	69.6 (29.7−163)	5.7 (3.2−10.0)
53	(3-phenyl-1-propyl)amino	2	*R*	126	MeCN	−14.8	$C_{35}H_{44}Cl_2N_4O_6$	185.6 (66.3−519)	11.7 (2.9−46.9)
54	naphth-1-ylamino	2	*S*	176	MeCN	+7.4[f]	$C_{36}H_{40}Cl_2N_4O_6$	2.0 (1.7−2.3)	0.7 (0.3−1.5)
55	naphth-1-ylamino	2	*R*	233	EtOH/H_2O, 3:1	+14.8[f]	$C_{36}H_{40}Cl_2N_4O_6$	8.5 (2.3−31.4)	1.7 (1.3−2.3)
56	naphth-1-ylamino	1	*S*	134	MeCN	−27.0[e]	$C_{35}H_{38}Cl_2N_4O_6$	21.5 (15.6−29.6)	1.9 (1.1−3.2)
57	naphth-2-ylamino	2	*R*	172	MeCN	+23.2[f]	$C_{36}H_{40}Cl_2N_4O_6$	65.7 (29.6−146)	3.2 (1.3−8.1)
58	*N*-(methylnaphth-1-yl)amino	2	*S*	122	MeCN	−13.5[e]	$C_{37}H_{42}Cl_2N_4O_6$	6.2 (3.0−13.0)	1.3 (0.8−2.2)
59	quinolin-8-ylamino	2	*S*	175	MeCN	−26.5[e]	$C_{35}H_{39}Cl_2N_5O_6$·HCl	10.8 (4.0−28.7)	5.4 (2.5−11.5)
60	quinolin-5-ylamino	2	*S*	183	iPrOH	−37.7	$C_{35}H_{39}Cl_2N_5O_6$·HCl	13.6 (8.2−22.7)	7.6 (1.6−35.5)
61	naphth-1-ylmethylamino	2	*S*	139	MeCN	−3.8[e]	$C_{37}H_{42}Cl_2N_4O_6$	6.3 (1.9−21.0)	0.9 (0.6−1.2)
62	tetralin-1-ylamino	2	*S*	210	MeCN	−15.8[f]	$C_{36}H_{44}Cl_2N_4O_6$	4.6 (1.7−12.7)	0.9 (0.3−2.8)
63	(5,6,7,8-tetrahydronaphth-1-yl)-amino	2	*S*	217	MeCN	−14.9[e]	$C_{36}H_{44}Cl_2N_4O_6$	5.7 (3.5−9.2)	1.6 (0.6−4.6)
64	5-indanylamino	2	*S*	146	MeCN	−28.3	$C_{35}H_{42}Cl_2N_4O_6$	72.7 (30.7−173)	11.8 (4.5−30.6)
65	1,2,3,4-tetrahydroquinolin-1-yl	2	*S*	202	MeOH	+35.3[e]	$C_{35}H_{42}Cl_2N_4O_6$	33.3 (16.9−65.8)	2.5 (1.6−3.9)
66	1,2,3,4-tetrahydroisoquinolin-2-yl	2	*S*	150	AcOEt	−3.7[e]	$C_{35}H_{42}Cl_2N_4O_6$	14.0 (5.8−33.6)	4.9 (2.1−11.8)
67	indolino	2	*S*	250	DMF/H_2O, 1:2	−21.5[e]	$C_{34}H_{40}Cl_2N_4O_6$	21.1 (7.4−59.9)	2.6 (1.9−3.5)

[a] Rectus configuration. [b] $[\alpha]_D$ in $CHCl_3$ ($c = 2$). [c,d] See corresponding footnotes to Table 1. [e] $[\alpha]_D$ in pyridine ($c = 2$). [f] $[\alpha]_D$ in MeOH ($c = 2$).

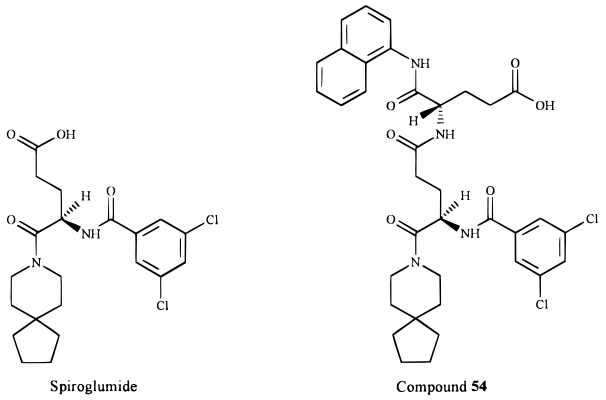


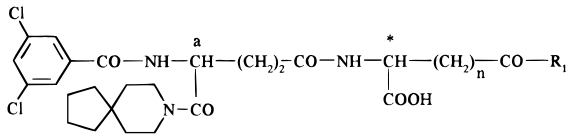
Figure 1. Schematic structure of spiroglumide and compound **54** (CR 2622).

and 5.4×10^{-8} M on the brain binding and parietal cells, respectively). Nevertheless the activity of these compounds is less interesting if compared with the activity described above for the derivatives of Tables 2 and 3, such as **28** and **54**.

54, the most potent among these spiroglumide derivatives, was further evaluated in order to establish if its interaction with CCK/gastrin receptor is competitive. Competition studies performed with [^{3}H]-*N*-methyl-*N*-leucine-CCK-8 in guinea pig brain tissue were determined in the presence and absence of different concentrations of **54** and analyzed according to Scatchard.[11] **54** inhibited competitively CCK-8 binding in guinea pig brain cortex: It reduced the slopes of the calculated regression lines without affecting the *x* intercepts of the plots, causing an increase in the dissociation constants (K_d) of CCK-8 for its receptors, without changing the maximum number of receptors (B_{max} of the Scatchard plot) (Figure 2). The control K_d value for CCK binding was 0.38 ± 0.05 nmol, and B_{max} was 68.9 ± 4.7 fmol/mg of protein. In the presence of **54**, the values for K_d became 0.94 (10 nmol inhibitor) and 1.62 (30 nmol inhibitor) nmol, respectively [one-way ANOVA: $F(2,5) = 71.5$ ($p = 0.003$)], and B_{max} was 97 ± 15% of control value [one-way ANOVA: $F(2,5) = 0.13$ NS].

Dose−response curves from Ca^{2+} mobilization studies in enriched gastric parietal cells of rabbit were performed to confirm the competitive antagonism described in the previous binding study. **54** produced a shift to the right of the gastrin dose−response curve without significant depression of maximal responses to gastrin, in a dose range of 1−10 nmol [Duncan test NS ($p = 0.05$

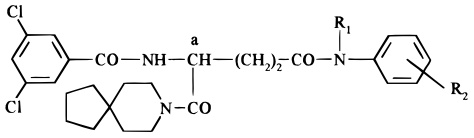
Table 4. Physical and Biological Properties of Spiroglumide γ-Amides of Glutamic or β-Amides of Aspartic Acid Derivatives



compd	R_1	n	conf (*)	mp, °C	recryst solvent	optical[b] rotation, deg	formula	IC_{50}, 10^{-8} mol: CCK_B (central)[c]	IC_{50}, 10^{-8} mol: gastrin (peripheral)[d]
68	benzylamino	1	*RS*	100	MeCN	+3.4	$C_{32}H_{38}Cl_2N_4O_6$	38.5 (22.3−66.3)	7.6 (5.5−10.7)
69	*N*-(methylbenzyl)amino	1	*R*	172	MeCN	+7.9	$C_{33}H_{40}Cl_2N_4O_6$	15.8 (5.9−42.3)	4.4 (1.5−12.8)
70	naphth-1-ylamino	1	*R*	148	MeCN	−6.3	$C_{35}H_{38}Cl_2N_4O_6$	6.1 (4.3−8.7)	2.3 (1.9−2.8)
71	*N*-(methylbenzyl)amino	2	*R*	113	MeCN	+6.1	$C_{34}H_{42}Cl_2N_4O_6$	7.8 (4.0−15.3)	5.5 (2.2−13.8)
72	naphth-1-ylamino	2	*R*	128	MeCN	+6.2	$C_{36}H_{40}Cl_2N_4O_6$	4.4 (2.0−9.4)	1.2 (0.8−1.9)

[a] Rectus configuration. [b] $[\alpha]_D$ in pyridine ($c = 2$). [c,d] See corresponding footnotes to Table 1.

Table 5. Physical and Biological Properties of Spiroglumide Amides with Aminobenzoic and Aminophenylacetic Acids



compd	R_1	R_2	mp, °C	recryst solvent	optical[b] rotation, deg	formula	IC_{50}, 10^{-8} mol: CCK_B (central)[c]	IC_{50}, 10^{-8} mol: gastrin (peripheral)[d]
73	H	4-carboxy	281	EtOH/H_2O, 3:1	+39.7[e]	$C_{28}H_{31}Cl_2N_3O_5$	176.3 (93.9−331)	56.8 (48.4−66.5)
74	H	3-carboxy	263	EtOH/H_2O, 5:2	+32.4[e]	$C_{28}H_{31}Cl_2N_3O_5$	130.0 (77.5−218)	16.1 (11.6−22.3)
75	H	2-carboxy	207	EtOH/H_2O, 2:1	+74.3	$C_{28}H_{31}Cl_2N_3O_5$	44.3 (24.1−81.5)	5.5 (3.4−8.9)
76	CH_3	4-carboxy	110	EtOH/H_2O, 3:1	−6.3	$C_{29}H_{33}Cl_2N_3O_5$	142.1 (77.6−260)	108.3 (48.2−243)
77	CH_3	2-carboxy	134	MeCN	−44.2	$C_{29}H_{33}Cl_2N_3O_5$	14.6 (9.3−22.9)	5.4 (4.3−6.7)
78	H	3-(carboxymethyl)	164	AcOEt/ETP,[f] 1:3	+17.7	$C_{29}H_{33}Cl_2N_3O_5$	21.2 (9.3−48.5)	9.7 (4.1−23.1)

[a] Rectus configuration. [b] $[\alpha]_D$ in $CHCl_3$ ($c = 2$). [c,d] See footnotes to Table 1. [e] $[\alpha]_D$ in pyridine ($c = 2$). [f] ETP = petroleum ether, 40−50 °C.

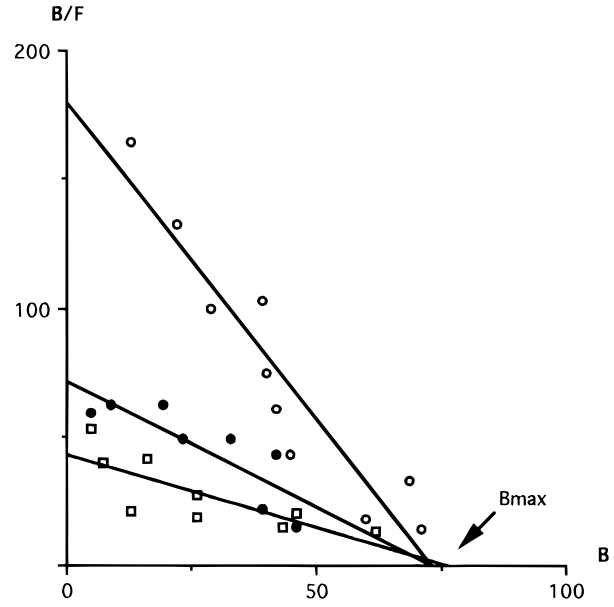


Figure 2. Competitive inhibition according to Scatchard of [^{3}H]-N-Me-N-Leu-CCK-8 binding in guinea pig brain cortex by **54**. Values are the mean of two determinations, each in duplicate: (○) no inhibitor, (●) 10 nmol inhibitor, and (□) 30 nmol inhibitor.

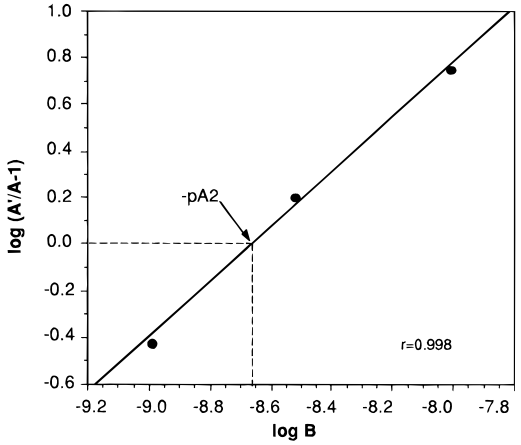


Figure 3. Effect of **54** on gastrin dose−response curves inducing Ca^{2+} mobilization in enriched gastric parietal cells of rabbit. The antagonism was evaluated by Schild plot. Each point represents the mean of two experiments, each in duplicate. Regression line: $\log(A'/A - 1) = -1.178(-\log B) + 10.2$ ($r = 0.998$); $pA_2 = 8.66$ (9.04−8.29) ($p = 0.05$).

level)]. The slope of the Schild plot did not differ significantly from unity (1.17), indicating that **54** interacts competitively also with stomach gastrin receptors [$pA_2 = 8.66$ (9.04−8.29), $p = 0.05$] (Figure 3).

To understand further the structure relationships between pentagastrin (Ala-Trp-Met-Asp-Phe) and **54**, a computer-assisted conformational analysis was carried out in order to determine the low-energy conformation of both the agonist and its antagonist. Therefore the structure of **54** was fitted to the structure of pentagastrin. For each molecule, the minimized structure energy (79.8 kcal/mol for pentagastrin and 37.8 kcal/mol for **54**) was maintained. **54** was superimposed on pentagastrin using a least-squares fit, after choosing the carboxylic

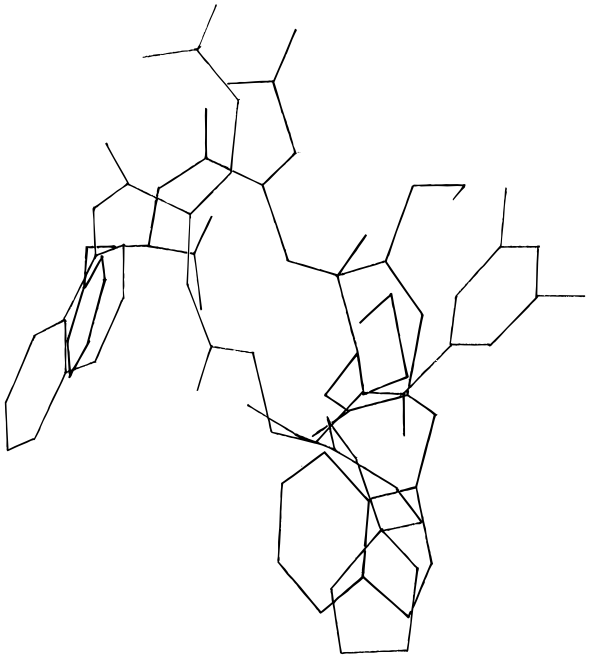


Figure 4. Computer superposition of **54** on pentagastrin (CCK_{29-33}) showing matching of the two carboxylic groups and of the azaspiro[4.5]decan-8-yl, 3,5-dichlorophenyl, and 1-naphthyl moieties of **54** with the indolyl, methionine, and phenylalanine moieties of pentagastrin, respectively: red, pentagastrin, and blue, **54**.

acid and three other significant hydrophobic groups for each of the two molecules.

Figure 4 shows the best-fitting superposition of **54** on pentagastrin, showing matching of carboxylic groups of the two molecules and an extensive overlap of the azaspiro group of the antagonist with the indolyl moiety of pentagastrin, of the 3,5-dichlorobenzamido moiety of **54** with the hydrophobic methionine moiety of pentagastrin, and of the 1-naphthyl group of **54** with the terminal phenylalaninamide group of pentagastrin, respectively. It is noteworthy that the distance calculated by computer between the two carbon atoms, selected as representatives for each of the three couples of hydrophobic moieties and for the two carboxylic acids of the two molecules, is on an average of 2.6 ± 0.7 Å.

In vivo, in the in situ perfused rat stomach, **54** reduced dose dependently the gastric acid secretion induced by pentagastrin infusion (30 μg/kg/h); its antigastrin ID_{50} value was 2 (1.1−3.6) mg/kg ($p = 0.05$ fiducial limits).

In order to assess the selectivity of their CCK_B/gastrin antagonism, **54** and compound **28** (CR 2767), the most effective gastrin antagonists among the series considered, were also tested to determine their affinity with the peripheral CCK_A receptor (binding to the rat pancreatic acini). The specific binding of [^{125}I]CCK-8 to rat pancreatic acini was inhibited by both **54** and **28** only at concentrations of about 2 orders of magnitude higher than their IC_{50} for inhibiting the specific binding of [^{3}H]-N-Me-N-Leu-CCK-8 in guinea pig brain cortex. The results are shown in Table 6.

Conclusion

Compound **54** is the most potent CCK_B antagonist among the acidic amido derivatives of spiroglumide synthesized and evaluated for their capacity to inhibit the binding of [^{3}H]-*N*-methyl-*N*-leucine-CCK-8 in guinea pig brain cortex and the gastrin-induced Ca^{2+} increase in rabbit parietal cells. This compound, as well as other representatives of this new series of spiroglumide derivatives, displayed nanomolar gastrin antagonism in vitro and was about 100 times more active than the parent compound.

Table 6. Comparison of CCK_A and CCK_B Inhibition by Spiroglumide Amido Acid Derivatives in Different Models (IC_{50}, nmol)

compd	[^{125}I]CCK-8 binding in rat pancreatic acini (CCK_A)	[^{3}H]-N-Me-N-Leu-CCK-8 binding in guinea pig brain cortex (CCK_B)[a]	A/B ratio
28 (CR 2767)	3400	57	60
54 (CR 2622)	7380	20	369
spiroglumide	13 500	1400	9.6

[a] Values drawn from Tables 1−3, respectively.

54 interacted competitively with both gastric and brain gastrin/CCK_B receptors. The compound was also strongly effective in vivo, by blocking the pentagastrin-stimulated acid secretion in the in situ perfused rat stomach. **54** demonstrated high selectivity for gastrin/CCK_B receptor. Its affinity at the peripheral pancreatic acini (CCK_A receptor) was in fact about 300 times lower than that shown at the central CCK_B/gastrin receptor.

The strong affinity for CCK_B/gastrin receptor demonstrated by **54** can be explained by its capacity to mimic the conformation of the terminal gastrin pentapeptide (CCK_{28-33}). Indeed, computer-assisted conformational analysis studies showed that the low-energy conformation of **54** gave a good matching on the low-energy pentagastrin structure, demonstrating the possibility of an extensive overlapping of both the three hydrophobic moieties and the carboxylic acids of the two molecules.

Further studies to evaluate the potential therapeutic use of **54** for treatment of anxiety disorders, linked to its antagonism for the central CCK_B receptor, will be reported in due course.

Experimental Section

Chemistry. The following procedures were adopted. ^{1}H NMR spectra were recorded at 60 MHz on a Varian EM360L or at 300 MHz on a Bruker CXP-300 instrument; infrared spectra were recorded on an Ati Mattson Genesis FT-IR series spectrophotometer using WinFirst software. Melting points were determined on a Buchi 535 apparatus and are incorrect. Elemental analyses were performed on a Perkin-Elmer elemental analyzer (CHNS/O 2400 Series II), and the analytical results varied within ±0.4% of the theoretical values unless otherwise noted. TLC was carried out using Merck silica gel GF_{254} plates with the following elution systems: A (isoamyl alcohol/acetone/water, 5:2:1) and B (benzene/methanol/glacial acetic acid, 45:8:8). Specific rotation was determined with a Jasco Dip-370 polarimeter at 589 nm in a 100 mm cell and at 2 g/100 mL. Diastereomeric excess for compounds **5**−**72** was higher than 95% and was determined by HPLC on an Alltech Econosphere C18 column, eluent, 75% MeOH in 10 mM KH_2PO_4 (pH 2.85), UV detection, $\lambda = 239$ nm; flow rate, 0.54 mL/min. Enantiomeric excess for compounds **1**−**4** and **73**−**78** was higher than 98% and was determined on an Econosphere C18 column after derivatizing the analyte with L-phenylalaninamide (Sigma) (mixed anhydride method);[10] UV detection, $\lambda = 239$ nm; gradient eluent, from 50% CH_3CN in 125 mM Na_2HPO_4 (pH 6.5) to 100% CH_3CN; flow rate, 1 mL/min.

Compounds **1**−**78** were obtained according to the described procedure for compound **54**. The amino acids used for the synthesis of compounds described in Tables 1, 2, and 5 were purchased from Sigma-Aldrich or Bachem Feinchemikalien AG; the amino acids used for compounds **22**−**24** and **30** were prepared according to the cited literature;[12] the amino acid

used for compound **28** was prepared according to the cited literature;[13] the isoglutamines or isoasparagines used for compounds described in Table 3 were prepared according to the cited literature;[8] the amine used for isoglutamine in the synthesis of **58** was prepared according to the cited literature;[14] the amino acids used for the products described in Table 4 were obtained according to the cited literature.[15] The starting material spiroglumide was prepared in our laboratories according to the cited literature.[8]

(*S*)-4-[[(*R*)-4′-[(3,5-Dichlorobenzoyl)amino]-5′-(8-azaspiro[4.5]decan-8-yl)-5′-oxopentanoyl]amino]-5-(1-naphthylamino)-5-oxopentanoic Acid (54). To a mechanically stirred solution of 15 g (0.034 mol) of (*R*)-4-(3,5-dichlorobenzamido)-5-(8-azaspiro[4.5]decan-8-yl)-5-oxopentanoic acid (spiroglumide) and 4.8 mL (0.035 mol) of triethylamine dissolved in 100 mL of THF at −10 °C was added a solution of 3.3 mL (0.035 mol) of ethyl chloroformate in 30 mL of THF dropwise. The stirring was continued at −10 °C for 15 min, and then a solution of 11.1 g (0.041 mol) of (*S*)-4-amino-5-(1-naphthylamino)-5-oxopentanoic acid, dissolved in a mixture of 50 mL of DMF, 35 mL of water, and 10 mL of triethylamine, was added dropwise. After 1 h at −10 °C and overnight at room temperature, the THF was evaporated under reduced pressure and the residual oil was dissolved in water and ethyl acetate. The organic layer was washed with HCl (2 N) and water, dried, and evaporated, resulting in a viscous oil which solidified under diisopropyl ether. The white solid was filtered to give 14.2 g (60% yield). An analytical sample was obtained by recrystallization from acetonitrile: mp 176 °C; TLC (A) R_f 0.60, (B) R_f 0.68; ^{1}H NMR ($CDCl_3$) 9.9, 8.2, 7.7, 4.9, 3.5, 2.5, 1.5; IR (KBr) 3270, 2935, 1717, 1663, 1627 cm^{-1}; $[\alpha]^{25}_D = +7.4°$ ($c = 2$, MeOH). The HPLC retention times of **54** and the *R*,*R*-epimer **55** were 16.8 and 13.8 min, respectively. Anal. ($C_{36}H_{40}Cl_2N_4O_6$) C, H, N.

Compounds **1**−**78**, shown in Tables 1−5, were synthesized with this procedure.

Biological Tests. Male adult guinea pigs, New Zealand white rabbits, and Sprague−Dawley rats were used. CCK-8(s) and pentagastrin were purchased from Peninsula Laboratories, human gastrin I was from Novabiochem (Switzerland), (N-Me,N-Leu^{28,31})CCK-8 was a gift of Dr. Hruby (University of Arizona, Tucson, AZ), [^{125}I](BH)CCK-8 (specific activity 2000 Ci/mmol) was from Amersham, [^{3}H](N-Me,N-Leu^{28,31})CCK-8 was from NEN, Ultima Gold was from Packard, fura-2/AM was from Calbiochem, Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], EGTA [ethylenebis(oxyethylenenitrilo)tetraacetic acid], collagenase type 1A, and trypan blue were purchased from Sigma, gentamicin sulfate and PBS (phosphate-buffered saline, Ca^{2+} and Mg^{2+} free) were from BioWhittaker, bovine serum albumin, fraction V (BSA), bestatin, and pronase were from Boehringer (Mannheim, Germany), bacitracin was from Aldrich, soybean trypsin inhibitor (SBTI) was from PL Biochemicals (St. Goar, Germany), and pentothal was from Abbott.

The compounds under investigation were dissolved as sodium salt in saline. Estimated concentrations or doses at 50% effect (IC_{50} or ID_{50}) and their $p < 0.05$ fiducial limits were calculated from the regression line of the percentage of maximum effect, discarding the 6% tails, on the logarithm of the concentration or of the dose.

Radioligand Binding Studies. Central CCK_B receptor binding assays were carried out on membranes of guinea pig cerebral cortex as previously described.[13] Briefly, tissue (about 1.5 g) was homogenized 1:10 (w/v) in ice-cold 10 mM Hepes (pH 7.4) and centrifuged at 4 °C for 15 min at 48000*g*. The pellet was suspended 1:20 (w/v) in tissue buffer (10 mM Hepes, 118 mM NaCl, 4.7 mM KCl, 5.0 mM $MgCl_2$, 1.0 mM EGTA, pH 7.4) and centrifuged as above. The final membrane pellet was suspended in tissue buffer to obtain the desired protein concentration, which was determined according to the method of Bradford.[16] Cortical membranes (0.35−0.4 mg of protein/tube), [^{3}H](N-Me,N-Leu^{28,31})CCK-8 (0.45 nM), and displacing agents were incubated for 150 min at 25 °C. All the components, other than cortical membrane suspension, were prepared in an assay buffer consisting of 1 mg/mL BSA, 50 μM bestatin, and 0.1 mg/mL bacitracin dissolved in tissue buffer. Bound radioligand was separated by rapid filtration on glass fiber filters (GFB, Whatman), pretreated for at least 1 h with assay buffer. Filtrates were washed three times with 4 mL of ice-cold 0.9% NaCl; filter discs were counted with 8 mL of Ultima Gold in a B1900 Tri-Carb (Packard) liquid scintillator, with 56% efficiency. Specific binding was determined as the difference between binding in the absence and presence of 1 μM CCK-8(s), representing on an average 65% of total binding. Inhibition binding constants were determined by nonlinear curve-fitting programs of McPherson.[17]

CCK_A receptor binding assays were carried out on rat pancreas by the collagenase method previously described.[18] Briefly, tissue (about 5 g) was extensively minced and dispersed in 30 vol of Krebs−Henseleit buffer (KHB) (118 mM NaCl, 25 mM $NaHCO_3$, 4.7 mM KCl, 1.2 mM NaH_2PO_4, 0.1 mM $CaCl_2$, 14 mM glucose, 0.1 mg/mL SBTI, 1% BSA) adjusted to pH 7.4; 0.5 mg/mL crude collagenase was added to the medium, and the mixture was continuously shaken and gassed with 95% O_2−5% CO_2. The resulting suspension was filtered with a nylon mesh (320 μm), layered over KHB containing 4% BSA, 0.5 mM $CaCl_2$, and 0.1 mg/mL SBTI, and centrifuged for 5 min at 132*g*. The final resulting pellet was suspended in 10 vol of Hepes−Ringer buffer, pH 7.4 (118 mM NaCl, 10 mM Hepes, 1.13 mM $MgCl_2$, 1.28 mM $CaCl_2$, 1% BSA). This preparation gave a final concentration of $1-3 \times 10^7$ cells/mL (assessed by light microscope with the aid of a Neuebauer chamber), which was diluted with buffer binding to about 5×10^6 cells/mL.

Acini (400 μL), tracer (55 000 dpm/tube), and displacing agents were incubated in 0.5 mL total volume in poly(propylene) tubes for 30 min at 37 °C. Then 1 mL of ice-cold assay buffer was added, and the tubes were centrifuged at 12500*g*. The supernatant was eliminated and the radioactivity associated to the pellet measured in a Packard 5000 γ-counter (80% efficiency). Nonspecific binding was estimated as 5 μM CCK-8 (on average 30%).

Intracellular Ca^{2+} Mobilization Studies. (a) Preparation of Isolated Rabbit Gastric Parietal Cells. Cell isolation was carried out following the collagenase/EDTA procedure already described[19] with minor modifications. Stomach was excised from male New Zealand white rabbits weighing about 2 kg [sacrificed by intravenous injection of 2 mL of penthotal (50 mg/mL)], opened along the smaller curvature, and rinsed with 0.15 mM calcium- and magnesium-free PBS (pH 7.4, 4 °C). The mucosa was scraped off, washed 3−4 times with PBS, and minced with scissors into small pieces. About 7.5 g of mucosa was placed into 37.5 mL of medium A (composition in mM: NaCl, 132; KCl, 5.4; $MgSO_4$, 1.2; NaH_2PO_4, 1; Na_2HPO_4, 5; $CaCl_2$, 1; Hepes, 25; plus glucose, 0.2%, BSA, 0.2%; and gentamicin, 50 μg/mL; pH 7.4) containing collagenase (0.25 mg/mL, 300−350 U/mg) and pronase (0.3 mg/mL). The incubation was carried out at 37 °C for 15 min in medium gassed with 95% O_2−5% CO_2. The supernatant was then discarded, and the fragments were rinsed with 2×25 mL of Ca^{2+}/Mg^{2+}-free medium A, added with EDTA (2 mM), and incubated in 25 mL of the same medium at room temperature. After a 10 min incubation, the supernatant was removed and discarded. The second digestion was done in medium A containing collagenase (0.25 mg/mL) at 37 °C under continuous gassing (95% O_2−5% CO_2) for 15 min. The third and final digestion was done in the same conditions for 20 min; the enzyme digestion was supported with mechanical dispersion by repetitive pipetting. After allowing the undigested fragments to settle, the supernatant was recovered, diluted four times with medium A, passed through a 200 μm nylon mesh, and centrifuged at 200*g* for 5 min. The pellet was resuspended in 40 mL of Earle's medium (containing in mM: NaCl, 116; KCl, 5.36; $MgSO_4$, 0.8; NaH_2PO_4, 1; $CaCl_2$, 1.8; Hepes, 10; plus glucose, 0.1%; and BSA, 0.2%; pH 7.4), passed through a 100 μm nylon mesh, centrifuged, resuspended again in the same medium, and passed through a 62 μm nylon mesh. Cell suspension was centrifuged at 200*g* for 5 min; the pellet was resuspended in 10 mL Earle's medium. The final cell suspension was constituted nearly by 20% parietal cells. Enrichment in parietal cells was obtained by counterflow centrifugal elutriation technique.[19] The apparatus for continu-

ous-flow elutriation consists of a centrifuge containing a rotor with four separation chambers (Curame 3000, Haereus) and a peristaltic pump fitted with loading equipment. Cell suspension (5 mL/chamber) was injected into the elutriator chambers. Cells were separated on the basis of varying sedimentation velocity in counterflow.[20] Purity was 81 ± 6% (mean ± SD), and viability was >95%, evaluated by exclusion of trypan blue. Normally (60−80) × 10^6 (72 ± 11 SD) cells were obtained from about 7.5 g of gastric mucosa.

(b) Calcium Measurement. Cytosolic free calcium concentration, $[Ca^{2+}]_i$, was monitored with the fluorescent calcium indicator fura-2, as described by Grynkiewicz et al.[21] Cells were incubated with fura-2/AM (final concentration 4 μM) for 20 min at 37 °C in Earle's medium. After loading, cell suspension was diluted 10 times with Earle's medium, centrifuged for 5 min at 200*g*, finally resuspended in 10 mL of Hepes-buffered saline (HBS composition in mM: NaCl, 145; $MgCl_2$, 1; KCl, 5; Hepes, 10; glucose, 10; pH 7.4) at (5−10) × 10^6cells/mL concentration, and kept at room temperature in the dark until use. Cell suspension (0.8 × 10^6cells/1.5 mL) was added to a fluorimeter cuvette thermostated at 37 °C under continuous stirring. Antagonists were added 1 min before agonists. Fluorescence recording was performed by dual excitation fluorimetry in a Perkin-Elmer fluorescence spectrometer (LS50B), using fast filter software package. Wavelengths were set at 340 and 380 nm for excitation and 505 nm for emission; data were collected every 0.2−0.4 s.

$[Ca^{2+}]_i$ was then calculated according to the equation of Grynkiewicz.[21] Dose−response curves were analyzed with Allfit program, non-linear curve-fitting, to obtain IC_{50} values by applying the four-parameter logistic equation.[22]

In the antagonism studies with **54**, the CCK_A antagonist devazepide (final concentration 0.1 μM in DMSO, 0.1%) was added 1 min before **54**. The pA_2 with 95% fiducial limits was calculated from the Schild plot,[23] using a computer statistics package developed by Tallarida and Murray.[24]

Antisecretory Activity in Rats. Fasted (24 h) male rats anesthetized with urethane were used. Gastric acid secretion was determined in the perfused rat stomach according to the method of Ghosh and Schild[25] with slight modifications. **54** was administered by iv bolus in triplicate, in four dose levels after 60 min from the beginning of pentagastrin infusion. The inhibition (%) of gastric acid secretion was calculated from the values of total acid output collected before (first 60 min of pentagastrin infusion) and after the administration of the substance (second 60 min of pentagastrin infusion).

Computer-Aided Design. The software used for superimposition of compound **54** on pentagastrin (CCK29-33) was MAD (Molecular Advanced Design; Oxford Molecular SA, X-Pole Ecole Polytechnique, F-91128 Palaiseau, Cedex, France) and run on an IBM RISC/6000 360 station. The optimization of low-energy conformations was conducted by Monte Carlo−Metropolis algorithm,[26] using a dynamic weighing of the randomly chosen rotation axes to be modified. The starting data set is minimized using a function that randomly selects the rotation axes. During the first part of the calculation, the "heaviest" rotation axes are favored, then the "lightest" ones (second part), and finally the "heaviest" ones again (third part). Hundreds of iterations until obtaining dE < 0.05 kcal/mol were performed for the optimization, according to Newton−Raphson. At the end of the calculations, **54** was superimposed on pentagastrin using a least-squares fit to minimize all the distances between the molecules. Four significant groups of each molecule were chosen: the carboxylic, the azaspiro, the 3,5-dichlorobenzamido, and the 1-naphthyl moieties for compound **54** and the carboxylic, the indole, the methionine, and the terminal phenylalaninamide moieties for pentagastrin, respectively.

Supporting Information Available: Tables containing the atomic coordinates, bond distances, bond angles, and torsion angles for computer-aided design for compound **54** and pentagastrin (21 pages). Ordering information is given on any current masthead page.

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