formed by replacing Sta with Leu-Leu (or Leu-Phe), are not substrates for pepsin or penicillopepsin but rather fairly good (1–10 μM) inhibitors of these enzymes.[75] Naming this type of inhibition should be deferred until a better understanding of the mode of inhibition is gained, but it should be apparent to the reader that these data complicate attempts to unravel aspartic proteinase catalytic mechanism from statine-derived inhibitor–enzyme complexes. Undoubtedly, these complexes serve to locate binding pockets within the enzyme active site, but the orientation of the catalytic groups to amide substrates may differ significantly from their positions during catalysis. How many other mechanism-based enzyme inhibitors are enhanced versions of non-transition-state complexes?

For aspartic proteinases, it appears the best models for observing the transition state or tetrahedral intermediate by physical methods will be found from nonhydrolyzed ketone pseudosubstrates, derived from established substrate sequences, that can be shown by ^{13}C NMR to be converted to tetrahedral adducts in the active site by an enzyme-catalyzed process. Hydrated carbonyl mimics, e.g., Sta^P **7** peptide derivatives will also be valuable models for tetrahedral intermediates when derived from established substrate sequences, but comparisons of K_i to K_s (or K_m) should be corrected for the possible contribution of entropic factors stabilizing EI complexes that cannot stabilize ES complexes.

Finally, the idea to use the transition-state analogue concept as a point of departure for designing novel enzyme inhibitors remains a valuable approach, especially when all reactants in the mechanism are considered. Clearly if an enzyme is constructed so as to force water and substrate to within covalent bond distances, then intrusion of added atoms on the inhibitor, even as small as a proton, must prevent attainment of geometry identical with that formed in transition state (cf. Figure 1B). This problem would appear to face all tetrahedral transition-state mimics modeled after hydrated trigonal bonds (Figure 1B, **37**, **38**). Detailed enzyme kinetics to establish the order of addition of substrates or release of products on either side of the anticipated transition state being modeled are vital to the rational design of mechanism-based inhibitors because this information establishes if collected-substrate or collected-product inhibitors are feasible. It would seem many challenges remain before this field is fully understood, but the potential benefits make the effort well worthwhile.

Acknowledgment. This work was supported in part by grants from the National Institutes of Health (AM20100) and from Merck Sharp and Dohme. I am indebted to my collaborators P. G. Schmidt, M. N. G. James, T. Hofmann, F. G. Salituro, M. W. Holladay, N. S. Agarwal, M. Kawai, J. Boger, and D. F. Veber for their valuable advice and numerous contributions to this research.

Registry No. **1**, 94162-03-5; **3**, 94162-04-6; **4**, 94162-05-7; **5**, 94162-06-8; **7**, 94162-07-9; **8**, 94162-08-0; **9**, 94162-09-1; **10**, 94162-10-4; **11**, 94162-11-5; **13a**, 94162-12-6; **13b**, 94233-98-4; 15a, 94162-13-7; **16a**, 94162-14-8; **16b**, 94233-99-5; **17**, 94162-15-9; **18**, 94162-16-0; **19**, 94162-17-1; **22**, 94162-18-2; **23**, 94162-19-3; **24**, 20777-68-8; **25**, 85139-13-5; **26**, 76166-65-9; **27**, 82131-82-6; **28**, 94162-22-8; **29**, 94162-23-9; **30**, 94162-24-0; **31**, 91417-07-1; **32**, 91291-45-1; **41**, 94162-20-6; **42**, 94162-21-7; aspartic protease, 78169-47-8; renin, 9015-94-5; porcine pepsin, 9001-75-6; penicillopepsin, 9074-08-2.

Articles

Structure–Activity Relationships of C-Terminal Tri- and Tetrapeptide Fragments That Inhibit Gastrin Activity

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Received May 2, 1984

A series of tri- and tetrapeptide derivatives, analogues of the gastrin C-terminal region with no phenylalanine residue, were synthesized. These peptides were tested for their ability to inhibit gastrin-stimulated acid secretion in vivo as well as binding of [^{125}I]-(Nle^{11})-HG-13 to gastric mucosal cell receptors in vitro. Most of the peptides tested exhibited gastrin antagonist activity in vivo and in vitro. Most active derivatives were 20–30 times more potent than the well-known gastrin antagonist derivatives proglumide and benzotript and had 20–200 times more binding affinity. The smallest fragment exhibiting antagonist activity was the tripeptide Boc-L-tryptophyl-L-methionyl-L-aspartic acid amide.

Early work on structure–activity relationships of gastrin, particularly those of Morley,[1] showed that all the diverse biological activities of the gastrins were found to reside in the C-terminal 14–17 portion of the molecule L-tryptophyl-L-methionyl-L-aspartyl-L-phenylalanine amide. Replacement of L-tryptophan, L-methionine, or L-phenylalanine residues led to agonists of varying potency, whereas even small changes at the L-aspartic acid residue resulted in inactive analogues. Many analogues of the type L-tryptophyl-L-methionyl-X-L-phenylalanine amide were prepared, but they were devoid of antisecretory activity. Some antigastrin peptides were recently proposed: Boc-

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Table I. Analytical and Physical Data of Peptide Derivatives Used in Biological Tests[a]

peptides	mp, °C	$[\alpha]_D$ (*c* 1, DMF), deg	R_f (C)	R_f (D)	anal. C, H, N
Boc-L-Trp-L-Met-L-Asp-NH_2 (**3**)	210 dec	−28.1	0.55	0.58	$C_{25}H_{37}N_5O_7S$
Boc-L-Trp-L-Leu-L-Asp-NH_2 (**4**)	226–228 dec	−35.4	0.54	0.55	$C_{26}H_{37}N_5O_7$
Boc-L-Trp-L-Ile-L-Asp-NH_2 (**5**)	223 dec	−32.9	0.53	0.47	$C_{26}H_{37}N_5O_7$
Boc-L-Trp-L-Nle-L-Asp-NH_2 (**6**)	220 dec	−23.4	0.50	0.43	$C_{26}H_{37}N_5O_7$
Boc-L-Trp-L-Ala-L-Asp-NH_2 (**7**)	238 dec	−17.7	0.36	0.33	$C_{23}H_{31}N_5O_7$
Boc-L-Trp-L-Phe-L-Asp-NH_2 (**8**)	220 dec	−33.2	0.50	0.43	$C_{29}H_{35}N_5O_7$
Boc-L-Trp-L-Pro-L-Asp-NH_2 (**9**)	215 dec	−31.8	0.34	0.33	$C_{25}H_{33}N_5O_7$
Boc-L-Trp-Gly-L-Asp-NH_2 (**10**)	150 dec	−24.5	0.25	0.23	$C_{22}H_{29}N_5O_7$
Z-L-Trp-L-Leu-L-Asp-NH_2 (**11**)	120–125	−9.5	0.47	0.33	$C_{29}H_{35}N_5O_7$
Boc-Gly-L-Trp-L-Met-L-Asp-NH_2 (**12**)	170 dec	−18.6	0.26	0.27	$C_{27}H_{38}N_6O_8S$
Boc-β-Ala-L-Trp-L-Met-L-Asp-NH_2 (**13**)	124–127	−28.6	0.32	0.40	$C_{28}H_{40}N_6O_8S$
Boc-β-Ala-L-Trp-L-Leu-L-Asp-NH_2 (**14**)	210 dec	−23.3	0.35	0.34	$C_{29}H_{42}N_6O_8$
Boc-β-Ala-L-Trp-L-Nle-L-Asp-NH_2 (**15**)	210 dec	−14.7	0.35	0.37	$C_{29}H_{42}N_6O_8$
Boc-L-Trp-L-Leu-L-Asp(Bzl)-NH_2 (**16**)	147–150	−28.9	0.87	0.91	$C_{33}H_{43}N_5O_7$
Boc-L-Trp-L-Met-L-Asp(Bzl)-NH_2 (**17**)	177–180	−24.8	0.88	0.92	$C_{32}H_{41}N_5O_7S$
Boc-L-Trp-L-Leu-L-Asp-OH (**18**)	150 dec	+11.2	0.39	0.23	$C_{26}H_{36}N_4O_8$
Boc-L-Trp-L-Leu-D-Asp-NH_2 (**19**)	185 dec	+4.8	0.55	0.50	$C_{26}H_{37}N_5O_7$
Boc-L-Trp-L-Leu-L-Asn (**20**)	170 dec	−10.5	0.28	0.14	$C_{26}H_{37}N_5O_7$
Boc-β-Ala-L-Trp-L-Leu-L-Asn (**21**)	148–155 dec	−14.0	0.24	0.11	$C_{29}H_{42}N_6O_8$
Boc-L-Trp-L-Leu-L-Glu-NH_2 (**22**)	116–117	−20.6	0.61	0.70	$C_{27}H_{39}N_5O_7$
Boc-L-Trp-L-Leu-L-Asp-Pip (**23**)	120–125 dec	−52.2	0.70	0.73	$C_{31}H_{45}N_5O_7$
Boc-L-Trp-L-Leu-L-Asp-$N(CH_3)_2$ (**24**)	175–180	−70.5	0.38	0.60	$C_{28}H_{41}N_5O_7$
Boc-L-Trp-L-Leu-L-Ala-NH_2 (**25**)	145–150	−16.1	0.68	0.84	$C_{25}H_{37}N_5O_5$

[a] Boc is *tert*-butyloxycarbonyl, Z is benzyloxycarbonyl, Pip is piperidino, solvents: C (AcOEt 9, MeOH 1, AcOH 0.5); D (acetone 95, methanol 5, acetic acid 1).

glycyl-L-tryptophyl-L-methionyl-glycine amide,[2] Boc-L-tryptophyl-L-methionyl-L-aspartyl-D-alanine amide[3] and NPS-gastrin,[4] but their antagonist activity was controversial.[5]

Gastrin and cholecystokinin have the same C-terminal tetrapeptide amide fragment (L-tryptophyl-L-methionyl-L-aspartyl-L-phenylalanine amide), which constitutes the active site. These two peptides exhibit the same spectrum of activities but are modulated in potency by different N-terminal extensions.[6,7] We recently reported the synthesis[8] and the biological activities[9] of a new member in the class of cholecystokinin receptor antagonists: (benzyloxycarbonyl)-L-tyrosyl(SO_3^-)-L-methionyl-glycyl-L-tryptophyl-L-methionyl-L-aspartic acid amide (CCK-27–32-NH_2). This peptide was the fragment of the C-terminal parent hormone, without the C-terminal phenylalanine residue. We also showed that CCK-27–32-NH_2 was able to antagonize the action of gastrin on gastric acid secretion in vivo[10] and concluded that the C-terminal phenylalanine residue was important for cholecystokinin- and gastrin-like activities but was not an essential requirement for binding to the respective receptors. This concept was supported by our finding that C-terminal gastrin fragments with no phenylalanine residue, e.g., (*tert*-butyloxycarbonyl)-L-tryptophyl-L-methionyl-L-aspartic acid amide, (*tert*-butyloxycarbonyl)-glycyl-L-tryptophyl-L-methionyl-L-aspartic acid amide, (benzyloxycarbonyl)-L-glutamyl-L-alanyl-L-tyrosyl-glycyl-L-tryptophyl-L-methionyl-L-aspartic acid amide, exhibited gastrin receptor antagonist activity as well as an inhibitory effect on gastrin-induced acid secretion in vivo.[11] Since the dipeptide (*tert*-butyloxycarbonyl)-L-methionyl-L-aspartic acid amide was devoid of any activity, we reported that the minimum fragment exhibiting this antagonist activity was the tripeptide (*tert*-butyloxycarbonyl)-L-tryptophyl-L-methionyl-L-aspartic acid amide. The finding of gastrin antagonist activity in a small molecule like a tripeptide prompted us to investigate a structure–activity relationship study on a scale which was until now not possible with polypeptide hormones. We attached value to such an investigation for two major reasons: it may contribute to our knowledge on the mode of action of gastrin, cholecystokinin, and parent hormones; and structure–activity relationship studies on gastrin receptor antagonists may enable a more rational approach to the design of simple molecule inhibitors.

Chemistry. The peptides prepared in this work (tri- and tetrapeptides) are summarized in Table I. As an example, only the synthesis of (*tert*-butyloxycarbonyl)-β-alanyl-L-tryptophyl-L-leucyl-L-aspartic acid amide, an active analogue of pentagastrin without the phenylalanine residue, will be described in detail. The other tri-and tetrapeptides were prepared according to the same procedure, unless otherwise stated. The dipeptide (*tert*-butyloxycarbonyl)-L-leucyl-(β-benzyl)-L-aspartic acid amide (Table II, **27**) was obtained by reaction of (*tert*-butyloxycarbonyl)-L-leucine with the trifluoroacetate salt of (β-benzyl)-L-aspartic acid amide in the presence of (benzotriazolyloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) as a coupling reagent.[12] Partial deprotection of the dipeptide **27** with trifluoroacetic acid (TFA) and coupling with (*tert*-butyloxycarbonyl)-L-tryptophan *p*-nitrophenyl ester[13] in the presence of 1-hydroxybenzotriazole[14] yielded (*tert*-butyloxycarbonyl)-

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Table II. Analytical and Physical Data of Protected Dipeptide Derivatives[a]

peptides	mp, °C	$[\alpha]_D$ (c 1, DMF), deg	R_f (A)	R_f (B)	anal. C, H, N
Boc-L-Met-L-Asp(Bzl)-NH_2 (**26**)	108–110	−37.5	0.70	0.78	$C_{21}H_{31}N_3O_6S$
Boc-L-Leu-L-Asp(Bzl)-NH_2 (**27**)	85–88	−39.3	0.73	0.84	$C_{22}H_{33}N_3O_6$
Boc-L-Ile-L-Asp(Bzl)-NH_2 (**28**)	152–154	−40.1	0.72	0.83	$C_{22}H_{33}N_3O_6$
Boc-L-Nle-L-Asp(Bzl)-NH_2 (**29**)	118–120	−38.6	0.70	0.83	$C_{22}H_{33}N_3O_6$
Boc-L-Ala-L-Asp(Bzl)-NH_2 (**30**)	54–55	−38.2	0.69	0.74	$C_{19}H_{27}N_3O_6$
Boc-L-Phe-L-Asp(Bzl)-NH_2 (**31**)	152–154	−28.5	0.72	0.83	$C_{25}H_{31}N_3O_6$
Boc-L-Pro-L-Asp(Bzl)-NH_2 (**32**)	57–61	−71.5	0.54	0.83	$C_{21}H_{30}N_3O_6$
Boc-Gly-L-Asp(Bzl)-NH_2 (**33**)	99–101	−15.0	0.33	0.78	$C_{28}H_{25}N_3O_6$
Z-L-Leu-L-Asp(But)-NH_2 (**34**)	135–137	−36.0	0.58	0.84	$C_{22}H_{33}N_3O_6$
Boc-L-Leu-L-Asp(Bzl)$_2$ (**35**)	58–60	−22.2	0.93	0.87	$C_{29}H_{38}N_2O_7$
Boc-L-Leu-D-Asp(Bzl)-NH_2 (**36**)	106–108	+19.3	0.58	0.79	$C_{22}H_{33}N_3O_6$
Boc-L-Leu-L-Asn-OBzl (**37**)	75–78	−15.6	0.75	0.79	$C_{22}H_{33}N_3O_6$
Boc-L-Leu-L-Glu(Bzl)-NH_2 (**38**)	99–104	−19.9	0.75	0.79	$C_{23}H_{35}N_3O_6$
Boc-L-Leu-L-Asp(Bzl)-Pip (**39**)	138–140	−72.8	0.90	0.92	$C_{27}H_{41}N_3O_6$
Boc-L-Leu-L-Asp(Bzl)-N$(CH_3)_2$ (**40**)	105–108	−74.1	0.81	0.88	$C_{24}H_{37}N_3O_6$
Boc-L-Leu-L-Ala-NH_2 (**41**)	137–142	−12.5	0.55	0.66	$C_{14}H_{27}N_3O_4$

[a] Boc is *tert*-butyloxycarbonyl; Z is benzyloxycarbonyl; Pip is piperidino; solvents: A (AcOEt); B (acetone 7, hexane 3).

Table III. Analytical and Physical Data of Protected Tripeptide Derivatives[a]

peptides	mp, °C	$[\alpha]_D$ (c 1, DMF), deg	R_f (A)	R_f (B)	anal. C, H, N
Boc-L-Trp-L-Ile-L-Asp(Bzl)-NH_2 (**42**)	173–177	−28.2	0.47	0.54	$C_{33}H_{43}N_5O_7$
Boc-L-Trp-L-Nle-L-Asp(Bzl)-NH_2 (**43**)	178–180	−22.8	0.48	0.52	$C_{33}H_{43}N_5O_7$
Boc-L-Trp-L-Ala-L-Asp(Bzl)-NH_2 (**44**)	118–121	−19.7	0.23	0.38	$C_{30}H_{37}N_5O_7$
Boc-L-Trp-L-Phe-L-Asp(Bzl)-NH_2 (**45**)	158–161	−32.2	0.51	0.54	$C_{36}H_{41}N_5O_7$
Boc-L-Trp-L-Pro-L-Asp(Bzl)-NH_2 (**46**)	105–108 dec	−47.6	0.31	0.56	$C_{32}H_{39}N_5O_7$
Boc-L-Trp-L-Gly-L-Asp(Bzl)-NH_2 (**47**)	100–105	−25.3	0.13	0.31	$C_{29}H_{35}N_5O_7$
Z-L-Trp-L-Leu-L-Asp(But)-NH_2 (**48**)	182–185 dec	−41.3	0.48	0.74	$C_{33}H_{43}N_5O_7$
Boc-L-Trp-L-Leu-L-Asp(Bzl)$_2$ (**49**)	137–138	−25.1	0.67	0.83	$C_{40}H_{48}N_4O_8$
Boc-L-Trp-L-Leu-D-Asp(Bzl)-NH_2 (**50**)	158–162	+5.6	0.48	0.72	$C_{33}H_{43}N_5O_7$
Boc-L-Trp-L-Leu-L-Asn-OBzl (**51**)	201–203 dec	−23.8	0.32	0.73	$C_{33}H_{43}N_5O_7$
Boc-L-Trp-L-Leu-L-Glu(Bzl)-NH_2 (**52**)	198–200	−19.0	0.64	0.73	$C_{34}H_{45}N_5O_7$
Boc-L-Trp-L-Leu-L-Asp(Bzl)-Pip (**53**)	97–101	−53.4	0.78	0.87	$C_{38}H_{51}N_5O_7$
Boc-L-Trp-L-Leu-L-Asp(Bzl)-N$(CH_3)_2$ (**54**)	110–112	−64.2	0.59	0.81	$C_{35}H_{47}N_5O_7$

[a] Boc is *tert*-butyloxycarbonyl; Z is benzyloxycarbonyl; Pip is piperidino; solvents: A (AcOEt); B (acetone 7, hexane 3).

Table IV. Analytical and Physical Data of Protected Tetrapeptide Derivatives[a]

peptides	mp, °C	$[\alpha]_D$ (c 1, DMF), deg	R_f (A)	R_f (B)	anal. C, H, N
Boc-Gly-L-Trp-L-Met-L-Asp(Bzl)-NH_2 (**55**)	152–156	−16.6	0.27	0.35	$C_{34}H_{44}N_6O_8S$
Boc-β-Ala-L-Trp-L-Met-L-Asp(Bzl)-NH_2 (**56**)	187–189	−14.8	0.17	0.32	$C_{35}H_{46}N_6O_8S$
Boc-β-Ala-L-Trp-L-Leu-L-Asp(Bzl)-NH_2 (**57**)	194–196	−25.5	0.30	0.81	$C_{36}H_{48}N_6O_8$
Boc-β-Ala-L-Trp-L-Nle-L-Asp(Bzl)-NH_2 (**58**)	195–200	−13.9	0.16	0.29	$C_{36}H_{48}N_6O_8$
Boc-β-Ala-L-Trp-L-Leu-L-Asn-OBzl (**59**)	168–171	−20.3	0.17	0.27	$C_{36}H_{48}N_6O_8$

[a] Boc is *tert*-butyloxycarbonyl; Z is benzyloxycarbonyl; Pip is piperidino; solvents: A (AcOEt); B (acetone 7, hexane 3).

L-tryptophyl-L-leucyl-(β-benzyl)-L-aspartic acid amide (**16**). Tripeptide **16** was treated with a mixture of trifluoroacetic acid/anisole (9:1) for 30 min and the resulting trifluoroacetate salt was allowed to react with (*tert*-butyloxycarbonyl)-β-L-alanine in the presence of BOP to yield (*tert*-butyloxycarbonyl)-β-alanyl-L-tryptophyl-L-leucyl-(β-benzyl)-L-aspartic acid amide (**57**). Removal of the benzyl protecting group was performed by hydrogenation in the presence of a 10% Pd/$BaSO_4$ catalyst to yield (*tert*-butyloxycarbonyl)-β-alanyl-L-tryptophyl-L-leucyl-L-aspartic acid amide (**14**). All peptides were identified by ^1H NMR spectrum, amino acid composition, and elemental analysis and all showed a single spot on TLC utilizing various solvent systems. Prior to use in biological tests, those peptides having a free carboxylic group were dissolved in 0.2 N NH_4OH and lyophilized. Physical and analytical data of peptides are reported in Table I–IV.

Biological Results and Discussion

The synthetic tri- and tetrapeptides were evaluated for their ability to inhibit gastrin-stimulated acid secretion in the in situ perfused rat stomach and binding of labeled (Nle-11)-human gastrin-13, ([^{125}I]-(Nle^{11})-HG-13), to its receptors. Gastric acid secretion was determined in vivo in urethane-anesthetized rats (ip) by perfused rat stomach method of Ghosh and Schild.[15] It was recently reported that some discrepancies were observed when this method was utilized.[5] However, these autoinhibitory effects were not found when the urethane was given intramuscularly.[16] For this reason, compounds **10**, **13**, **14**, **17**, and **23** were tested as antisecretory compounds by the in situ perfused rat stomach method in rats anesthetized with urethane, im and ip. We did not find any difference in the ED_{50} of these compounds, regardless of the mode of administration of urethane. Since both the dipeptides Boc-Met-Asp-NH_2 (**1**) and Boc-Leu-Asp-NH_2 (**2**) were devoid of agonist and antagonist activity and because the tryptophan residue has been found to be of primary importance for receptor recognition,[1] the tryptophan residue has been included in all of the tri- and tetrapeptides we have synthesized in this study.

N-Acylation by *tert*-butyloxycarbonyl (Boc) or benzyloxycarbonyl (Z) groups seems to slightly affect antagonist activity as shown by the results obtained with compounds **4** and **11**. The compound (*tert*-butyloxycarbonyl)-L-tryptophyl-L-leucyl-L-aspartic acid amide (**4**) and (benzyloxycarbonyl)-L-tryptophyl-L-leucyl-L-aspartic acid amide (**11**) both inhibited gastrin-stimulated acid secretion: ED_{50} =

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Table V. Antagonist Activity and Inhibition of [^{125}I]-(Nle^{11})-HG-13 Binding to Its Receptors by Tri- and Tetrapeptides[a]

peptides	IC_{50}, μM	ED_{50}, μM/kg
Boc-Trp-Met-Asp-NH_2 (**3**)	30	14
Boc-Trp-Leu-Asp-NH_2 (**4**)	50	15
Boc-Trp-Ile-Asp-NH_2 (**5**)	50	25
Boc-Trp-Nle-Asp-NH_2 (**6**)	30	14
Boc-Trp-Ala-Asp-NH_2 (**7**)	400	30
Boc-Trp-Phe-Asp-NH_2 (**8**)	30	26.5
Boc-Trp-Pro-Asp-NH_2 (**9**)	100	15
Boc-Trp-Gly-Asp-NH_2 (**10**)	1000	60
Z-Trp-Leu-Asp-NH_2 (**11**)	40	38
Boc-Gly-Trp-Met-Asp-NH_2 (**12**)	25	8
Boc-β-Ala-Trp-Met-Asp-NH_2 (**13**)	20	7.5
Boc-β-Ala-Trp-Leu-Asp-NH_2 (**14**)	15	11.5
Boc-β-Ala-Trp-Nle-Asp-NH_2 (**15**)	70	13
Boc-Trp-Leu-Asp(Bzl)-NH_2 (**16**)	ND	inactive
Boc-Trp-Met-Asp(Bzl)-NH_2 (**17**)	ND	inactive
Boc-Trp-Leu-Asp-OH (**18**)	30	27.3
Boc-Trp-Leu-D-Asp-NH_2 (**19**)	35	17
Boc-Trp-Leu-Asn (**20**)	70	19
Boc-β-Ala-Trp-Leu-Asn (**21**)	70	16.4
Boc-Trp-Leu-Glu-NH_2 (**22**)	20	11
Boc-Trp-Leu-Asp-Pip (**23**)	30	8
Boc-Trp-Leu-Asp-$N(CH_3)_2$ (**24**)	100	10
Boc-Trp-Leu-Ala-NH_2 (**25**)	150	150
benzotript	250	170
proglumide	2500	350

[a] Pip is piperidino.

15 μM/kg for **4** and ED_{50} = 38 μM/kg for **11**.

Many changes can be made at the methionine position without loss of antagonist activity. This antagonist effect is not impaired by replacing the sulfur atom by methylene (norleucine analogue **6**, ED_{50} = 14 μM/kg) or by branching side chains (leucine and isoleucine analogues **4**, ED_{50} = 15 μM/kg, and **5**, ED_{50} = 25 μM/kg).

Antagonist activity decreased as the side chain is shortened (alanine analogue **7**, ED_{50} = 30 μM/kg) or suppressed (glycine analogue **10**, ED_{50} = 60 μM/kg). Addition of an aromatic moiety to the side chain (phenylalanine analogue **8**) or replacing the methionine residue by a proline residue (**9**) did not dramatically affect the antagonist activity. These observations support the results reported by Morley[1] on the structure–function relationship studies of gastrin-like peptides with agonist activity.

However, different results were obtained when the aspartic acid residue was replaced or modified. Most of the amino acid substitutions shown in Table V yielded compounds with little or no antagonist activity. Masking the β-carboxylic function of the aspartic residue (by a benzyl group, compounds **16** and **17**) lead to inactive derivatives. Replacement of the aspartyl residue by an asparaginyl residue led to compounds of weaker activity (**20** and **21**). Replacing the aspartic acid by a glutamic acid (L-glutamyl analogue **22**) produced active antagonist derivatives (ED_{50} = 11 μM/kg) as did the replacement of L-aspartic by a D-aspartyl residue (**19**, ED_{50} = 17 μM/kg). Suppression of the carboxylic side chain function (alanine analogue **25**) produced inactive compounds. Suppression of the terminal amide group (**18**) lead to compounds of weaker activity (ED_{50} = 27 μM/kg).

Nevertheless, a limited number of substitutions seems to be allowed on the nitrogen atom of the terminal amide. Thus, the piperidino and the *N,N*-dimethyl analogues (**23** and **24**) are very active antagonist compounds: ED_{50} = 8 μM/kg for **23** and ED_{50} = 10 μM/kg for **24**.

Extension of the N-terminal in the peptide chain of the active tripeptides by a glycine or even better by a β-alanine (compounds **12–15**) lead to better antagonist derivatives by at least a twofold order of magnitude: ED_{50}= 8 μM/kg for **12** and **13**, and ED_{50} = 12 μM/kg for **14** and **15**. The tri- and tetrapeptides presented exhibit antagonist activity and did not show any agonist activity even at doses as high as 50 mg/kg. In comparison to proglumide[17] or benzotript,[18] two well-known gastrin inhibitors, (*tert*-butyloxycarbonyl)-L-tryptophyl-L-leucyl-L-aspartic acid amide (**4**) is 10–20 times more potent when the same in vivo model is used. The biological results obtained on the inhibition of gastrin-stimulated acid secretion, in vivo, are in accordance with those observed in vitro on inhibition of binding by the peptides of labeled (Nle^{11})-HG-13 to its receptors. The biological event correlated with the inhibition of binding (Table V), except that, a decrease in the in vivo antagonist activity results in a greater decrease of inhibition of binding of labeled (Nle^{11})-HG-13 to gastrin receptors.

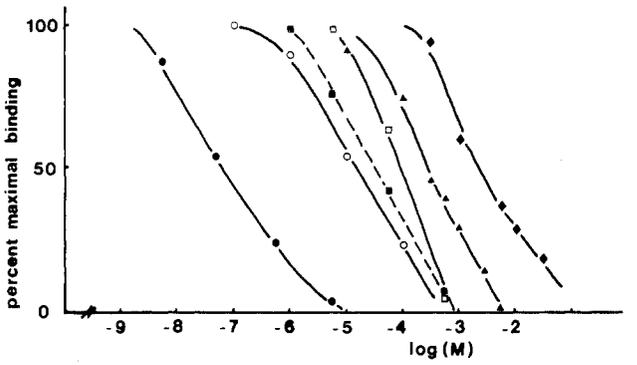

Figure 1. Competitive inhibition of [^{125}I]-(Nle^{11})-HG-13 specific binding to gastric mucosal cells by different peptides. Twenty picomoles of [^{125}I]-(Nle^{11})-HG-13 was incubated with gastric cells (5×10^6 per mL) for 30 min at 37 °C in the presence of various concentration of peptides, and then centrifuged and the radioactivity associated with the cell pellet was counted. Nonsaturable binding was determined in the presence of 1×10^{-6} M unlabeled (Nle^{11})-HG-13, (●) Boc-β-Ala-Trp-Met-Asp-Phe-NH_2 (pentagastrin); (○) Boc-β-Ala-Trp-Met-Asp-NH_2, (■) Boc-Trp-Met-Asp-NH_2, (□) Boc-Trp-Pro-Asp-NH_2, (▲) benzotript, (♦) proglumide.

The tetrapeptide (*tert*-butyloxycarbonyl)-β-alanyl-L-tryptophyl-L-leucyl-L-aspartic acid amide (**14**), representing pentagastrin without the C-terminal showed a decrease in the apparent affinity for gastrin receptors with an antagonist activity (Table V and Figure 1). This compound is 20–30 times more potent than proglumide or benzotript and has 20–200 times more binding affinity. All the results are summarized in Table V and Figure 1.

Deletion of the C-terminal phenylalanyl residue in the sequence of gastrin lead to a new class of peptides exhibiting gastrin antagonist activity. The minimum fragment having this antagonist activity is the tripeptide (*tert*-butyloxycarbonyl)-L-tryptophyl-L-methionyl-L-aspartic acid amide. It was used as a model for a structure–activity relationship study. According to previous results (particularly Morley's results[1]), we chose not to modify or replace the tryptophyl residue. The methionine, however, can be replaced with no significant loss of antagonist activity and an alkyl side chain seems to be beneficial. In our experiments, the aspartyl residue does accept some modifications without losing antagonist activity: the carboxylic side chain has to be present and its spacial position is of some importance. Removal of the terminal

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amide group, leading to the parent acid compound, produced less active molecules. However, substitutions on the nitrogen atom of the C-terminal amide group are allowed.

Experimental Section

All capillary melting points were determined on a Buchi apparatus and are reported uncorrected. Thin-layer chromatography (TLC) experiments were carried out on Merck silica gel GF_{254} plates. Column chromatography was on Merck silica gel, 60–230 mesh, ASTM. Elemental analyses were performed by "Le Service Central de Microanalyse du CNRS" de Montpellier. ^1H NMR spectra were performed by "Le Service de RMN du Centre de Pharmacologie-Endocrinologie de Montpellier" on a 360-MHz Brucker instrument. Abbreviations used were as follows: DMF, dimethylformamide; BOP (benzotriazolyloxy)tris(dimethylamino)phosphonium hexafluorophosphate; DIEA, diisopropylethylamine; TFA, trifluoroacetic acid; Boc, *tert*-butyloxycarbonyl; Z, benzyloxycarbonyl.

Chemistry. All the peptides described in this work were synthesized according to the general procedures detailed in this section for the preparations of compounds 4 and 16.

(*tert*-Butyloxycarbonyl)-L-leucyl-β-benzyl-L-aspartic Acid Amide (27). To a solution of β-benzyl-L-aspartic acid amide trifluoroacetate (1.91 g, 5.7 mmol) in DMF (10 mL) cooled in an ice water bath were added (*tert*-butyloxycarbonyl)-L-leucine *p*-nitrophenyl ester[19] (1.97 g, 5.6 mmol), 1-hydroxybenzotriazole[14] (0.86 g, 5.6 mmol), and diisopropylethylamine (2.06 mL, 12 mmol). After 30 min, the solution was allowed to warm at room temperature and was stirred overnight. The solvent was removed in vacuo and the residue was dissolved in ethyl acetate (200 mL), washed with a saturated sodium bicarbonate solution (2 × 60 mL), water (2 × 50 mL), a 10% citric acid solution (2 × 50 mL), and water (2 × 50 mL). The organic layer was dried over sodium sulfate and then concentrated in vacuo. The residue, triturated twice with a mixture of ethyl acetate/ether (1:9, v/v) and then with ether, gave a white powder: yield 80%, 1.95 g; R_f (A) 0.73, R_f (B) 0.84; mp 85–88 °C; $[\alpha]_D$ −39.3° (*c* 1, DMF). Anal. ($C_{22}H_{33}N_3O_6$) C, H, N.

(*tert*-Butyloxycarbonyl)-L-tryptophyl-L-leucyl-β-benzyl-L-aspartic Acid Amide (16). Compound 27 (2.18 g, 5 mmol) was partially deprotected in trifluoroacetic acid (10 mL) for 30 min. After that time, ether (150 mL) was added and the precipitate was collected by filtration, washed several times with ether, and dried in vacuo. This trifluoroacetate salt was dissolved in DMF (10 mL) and treated with (*tert*-butyloxycarbonyl)-L-tryptophan *p*-nitrophenyl ester[13] (2.34 g, 5.5 mmol), 1-hydroxybenzotriazole (0.86 g, 5.6 mmol), and diisopropylethylamine (2.06 mL, 12 mmol). After standing overnight at room temperature, the reaction mixture was treated as described for 27 and purified on a column of silica gel with a mixture of ethyl acetate/methanol (9:1) as eluent: yield 84% (2.6 g); R_f (A) 0.87, R_f (B) 0.91; mp 140–145 °C; $[\alpha]_D$ −28.9° (*c* 1, DMF). Anal. ($C_{33}H_{43}N_5O_7$) C, H, N.

(*tert*-Butyloxycarbonyl)-L-tryptophyl-L-leucyl-L-aspartic Acid Amide (4). Obtained by hydrogenation of 16 (1.86 g, 3 mmol) in 95% ethanol (100 mL) in the presence of a 10% Pd/C catalyst (0.1 g). After 6 h, the reaction mixture was filtered followed by addition of DIEA (1 mL). The solvent was concentrated in vacuo and the resulting residue dissolved in a 10% bicarbonate solution. The aqueous layer was extracted with ethyl acetate (50 mL) and ether (50 mL) and cooled to 5 °C. Acidification with citric acid gave a precipitate which was filtered, rinsed several times with ether, and dried in vacuo over phosphorus pentoxide: yield 70% (1.1 g); R_f (C) 0.54, R_f (D) 0.55; mp 226–228 °C; $[\alpha]_D$ −35.4° (*c* 1, DMF). Anal. ($C_{26}H_{37}N_5O_7$) C, H, N. The peptide was dissolved in 0.2 N NH_4OH and lyophilized before being used in biological tests. Hydrogenations of methionine-containing peptides were performed according to a procedure already described.[20]

(*tert*-Butyloxycarbonyl)-β-alanyl-L-tryptophyl-L-leucyl-β-benzyl-L-aspartic Acid Amide (57). After partial deblocking of 16 (1.24 g, 2 mmol) by TFA (10 mL) as described above, acylation was carried out in dimethylformamide (10 mL) with (*tert*-butyloxycarbonyl)-β-alanine (0.38 g, 2.2 mmol) and BOP (0.97 g, 2.2 mmol) as coupling reagent[12] in the presence of diisopropylethylamine (0.86 mL, 5 mmol). After standing overnight at room temperature, the reaction mixture was treated as described previously for 16 and purified on a silica gel column with a mixture of ethyl acetate/methanol (9:1) as eluent: yield 81% (1.12 g); R_f (A) = 0.30, R_f (B) 0.81; mp 194–196 °C; $[\alpha]_D$ −25.5° (*c* 1, DMF). Anal. ($C_{36}H_{48}N_6O_8$) C, H, N.

(*tert*-Butyloxycarbonyl)-β-alanyl-L-tryptophyl-L-leucyl-L-aspartic Acid Amide (14). Compound 57 (0.7 g, 1 mmol) was hydrogenated as described previously to yield 14 [0.42 g, 70%; mp 210 °C dec; $[\alpha]_D$ −23.3° (*c* 1, DMF); R_f (C) 0.35, R_f (B) 0.34; Anal. ($C_{29}H_{42}N_6O_8$) C, H, N], dissolved in 0.2 N NH_4OH and lyophilized to yield a white powder, before being used for biological tests. Hydrogenations of methionine-containing compounds were performed according to a procedure already described.[20]

Biological Tests. Gastric Acid Secretion. Gastric acid secretion was determined in vivo in the reperfused rat stomach according to the method of Ghosh and Schild.[15] The gastric pouch of an anesthetized rat (urethane ip or im for some compounds) was continuously washed at 30 °C with a propionate–succinate solution. The cumulative pH was recorded with time and used as an index of acid secretion. Synthetic (Leu^{15})-human gastrin I (gift from Professor E. Wunsch, Max Plank Institute, Munchen) was dissolved in 0.9% NaCl and was given as a bolus injection. The amount of H^+ secreted was determined by the pH difference between stimulated and basal recorded traces. Eighty picomoles of gastrin was usually employed as stimulant. The inhibitory effect of synthetic peptides was measured after the simultaneous bolus injection of the compounds (in water solution) and gastrin. The amount of H^+ secreted in the presence of various doses of the peptides was reported relative to the amount of H^+ secreted after gastrin alone and expressed as percent of inhibition. The mean H^+ secretion after gastrin injection was 203 ± 29 μmol of H^+/nmol of peptide (number of experiments = 17).

Binding Studies. Gastric cell isolation was carried out by using the collagenase/EDTA procedure as previously described.[21] Briefly, fundic mucosa was scraped and tissues were chopped into small cubes and then dispersed in medium A (132 mM NaCl, 5.4 mM KCl., 5 mM Na_2HPO_4, 1 mM NaH_2PO_4, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 25 mM Hepes, 0.2% glucose, 0.2% bovine serum albumin, 0.02% phenol red, pH 7.4) (gassed O_2/CO_2) containing 0.30 mg/mL of collagenase. After 15 min of incubation at 37 °C, tissue fragments were allowed to settle and the medium was discarded. The fragments were washed in Ca^{2+}-free medium A containing 2 mM EDTA and incubated in the same medium for 10 min. The fragments were transferred to medium A containing 0.30 mg/mL of fresh collagenase and incubated for 15 min at 37 °C with continuous gassing (O_2/CO_2). The cell suspension was centrifuged for 15 min at 200*g* and washed twice with medium A. This procedure gave about 2×10^7 cells/g of wet mucosa with 95% viability (tryptan blue exclusion). The mixed population contained 45% parietal cells. (Nle^{11})-HG-13 was iodinated following a modification of the chloramine T procedure already described.[21] After purification by DE-52 ion exchange chromatography, the monoiodinated peptide with full biological activity was obtained. Specific gastrin binding was determined by incubation in medium B (Earle's balanced salt medium without bicarbonate and containing 10 mM Hepes and 0.2% BSA, pH 7.4) of 20 pM labeled (Nle^{11})-HG-13 (≃ 40000 cpm/mL) for 30 min at 37 °C with 5×10^6 cells/mL ± various concentrations of peptides or unlabeled (Nle^{11})-HG-13. Nonsaturable binding was determined as the amount of radioactivity associated with cells in the presence of 1×10^{-6}M of unlabeled (Nle^{11})-HG-13 and substracted from total binding for the determination of the specific binding.

Acknowledgment. This work was supported by grants from "Le Centre National de la Recherche Scientifique"

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and PIRMED. We also acknowledge SANOFI Recherche for their assistance in this program and A. Turner for revision of the language of the manuscript.

Registry No. **3**, 92762-82-8; **4**, 94236-27-8; **5**, 94236-28-9; **6**, 94236-29-0; **7**, 94236-30-3; **8**, 94236-31-4; **9**, 94236-32-5; **10**, 94236-33-6; **11**, 94236-34-7; **12**, 92762-84-0; **13**, 94236-35-8; **14**, 94236-36-9; **15**, 94236-37-0; **16**, 94236-38-1; **17**, 92762-81-7; **18**, 94236-39-2; **19**, 94236-40-5; **20**, 94236-41-6; **21**, 94236-42-7; **22**, 94236-43-8; **23**, 94236-44-9; **24**, 94236-45-0; **25**, 94236-46-1; **26**, 92762-79-3; **27**, 94236-47-2; **28**, 94236-48-3; **29**, 94236-49-4; **30**, 94236-50-7; **31**, 94236-51-8; **32**, 94249-75-9; **33**, 94249-76-0; **34**, 88499-15-4; **35**, 32949-42-1; **36**, 94236-52-9; **37**, 94236-53-0; **38**, 94236-54-1; **39**, 94236-55-2; **40**, 94236-56-3; **41**, 94236-57-4; **42**, 94236-58-5; **43**, 94236-59-6; **44**, 94236-60-9; **45**, 94236-61-0; **46**, 94236-62-1; **47**, 94236-63-2; **48**, 94236-64-3; **49**, 94236-65-4; **50**, 94236-66-5; **51**, 94236-67-6; **52**, 94236-68-7; **53**, 94236-69-8; **54**, 94236-70-1; **55**, 92762-83-9; **56**, 94236-71-2; **57**, 94236-72-3; **58**, 94236-73-4; **59**, 94236-74-5; BOC-Leu, 13139-15-6; Asp(Bzl)-NH_2·TFA, 92762-94-2; BOC-Trp-PNP, 15160-31-3; Trp-Leu-Asp(Bzl)-NH_2·TFA, 94236-76-7; BOC-β-Ala, 3303-84-2; gastrin, 9002-76-0.

Synthesis of New Polyoxin Derivatives and Their Activity against Chitin Synthase from *Candida albicans*

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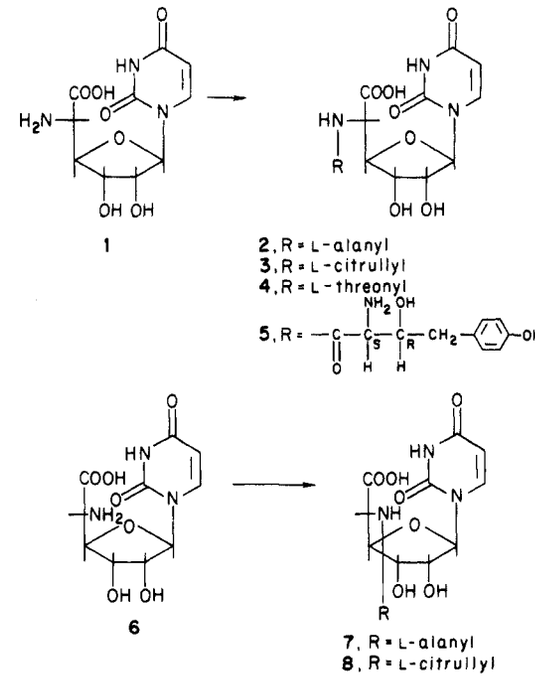
Two analogues of L-alanylpolyoxin C with a modified peptide bond were synthesized and tested for inhibition of chitin synthase in *Candida albicans*. N-Methylation of the peptide bond (compound **13**) or the replacement of it by NH_2CH_2 (compound **9**) led to loss of activity in the enzyme assay. A novel analogue (compound **5**) of nikkomycin was synthesized from uracil polyoxin C and (2*S*,3*R*)-3-hydroxyhomotyrosine, a component of echinocandin C. Despite high activity in the chitin synthase assay, **5** had no inhibitory effect on cells of *C. albicans*.

Polyoxins[1] and nikkomycins[2-4] (neopolyoxins[5-7]) are peptidyl nucleoside antibiotics with marked activity against phytopathogenic fungi. They inhibit the enzyme chitin synthase,[1,8,9] which catalyzes the final step in the biosynthesis of chitin, and bear a certain structural resemblance to uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc), the natural substrate of this enzyme. By virtue of this specific mode of action against an essential fungal enzyme, they are potentially ideal agents for the treatment of human fungal diseases, in addition to their existing agricultural role.[1]

Although the chitin synthase of human pathogenic fungi, such as *Candida albicans*, is highly sensitive to polyoxins in cell-free systems (K_i values in the micromolar range[10]), the growth of intact cells is inhibited at only high concentrations (millimolar range) of the antibiotic.[11] No completely satisfactory explanation for this discrepancy has yet been advanced. The most plausible reasons include a failure to be transported into the cell, or inactivation inside the cell, for example by cleavage of the peptide bond. Recent investigations,[12] published since the completion of our studies, have shown that certain tripeptidyl polyoxins do undergo cleavage at the peptide bond when incubated with cell extracts of *Candida albicans*. Moreover, metabolic degradation by peptidases of the host must be taken into consideration as a possible drawback of application to man.

These considerations led us to prepare polyoxin analogues with a modified peptide bond and to study their biological activity. As target structures we chose a dipeptide which features a peptide bond stabilized by a

Scheme I



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