(38) W. H. Sawyer, *Endocrinology*, **63**, 694 (1958).
(39) H. O. Schild, *Br. J. Pharmacol. Chemother.*, **2**, 189 (1947).
(40) J. Führ, J. Kaczmarczyk, and C. D. Krüttgen, *Klin. Wochschr.*, **33**, 729 (1955).
(41) B. F. Gisin, *Helv. Chim. Acta*, **56**, 142 (1973).
(42) E. Kaiser, R. L. Colescott, C. D. Bossinger, and P. I. Cook, *Anal. Biochem.* **34**, 595 (1970).
(43) J. Meienhofer, A. Trzeciak, R. T. Havran, and R. Walter, *J. Am. Chem. Soc.*, **92**, 7199 (1970).
(44) G. L. Ellman, *Arch. Biochem. Biophys.*, **82**, 70 (1959).
(45) S. Moore, *J. Biol. Chem.*, **238**, 235 (1963).
(46) A. M. Felix, C.-T. Wang, A. A. Liebman, C. M. Delany, T. Mowles, B. A. Burghardt, A. M. Charnecki, and J. Meienhofer, *Int. J. Pept. Protein Res.*, **10**, 299 (1977).
(47) For details of amino acid analysis procedure for Δ^3-Pro, see ref 7b.
(48) D. B. Hope, V. V. S. Murti, and V. du Vigneaud, *J. Biol. Chem.*, **237**, 1563 (1962).
(49) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

Tripeptide Analogues of Melanocyte-Stimulating Hormone Release-Inhibiting Hormone (Pro-Leu-Gly-NH_2) as Inhibitors of Oxotremorine-Induced Tremor

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Fourteen di- and tripeptide analogues of MIF, Pro-Leu-Gly-NH_2, have been synthesized and assayed for inhibition of oxotremorine-induced tremor. Replacement of Pro by HCO-Pro or cyclopentanecarboxylic acid gave inactive analogues, while some peptides of the general structure <Glu-Leu-Gly-NR_1R_2 were highly active. Thus, $R_1 = C_3H_8$ and R_2 = H gave 4 times the activity of MIF, $R_1 = i\text{-}C_3H_8$ and R_2 = H gave 13 times the activity of MIF, and $R_1 = R_2 = CH_3$ gave 29 times the activity of MIF. *cyclo*(-Pro-Leu-), Pro-Lys-Gly-NH_2, and Pro-Arg-Gly-NH_2 had no activity. Apparently, small modifications in the structure of MIF can yield highly active analogues with potential clinical value, e.g., in the treatment of Parkinson's disease or mental depression.

Prolylleucylglycine amide (MSH release-inhibiting hormone, MIF, melanostatin) is a peptide with pronounced direct effects on the central nervous system. Several studies have shown that MIF potentiates the behavioral effects of L-Dopa[1,2] or apomorphine,[3] reverses the central and peripheral effects of oxotremorine,[4,5] antagonizes the sedative effects of deserpidine,[6] attenuates puromycin-induced amnesia,[7] facilitates morphine dependence,[8] and influences learning and spontaneous behavior in several species.[3,9-11] The first four effects are typical for drugs active against Parkinson's disease. In agreement with these data, MIF has been shown to ameliorate the symptoms of clinical parkinsonism,[12-14] either alone or in conjunction with L-Dopa. Increasing experimental evidence also points to a beneficial effect of MIF in mental depression.[15-17]

However, large doses of peptide are required to bring about clinical effects, which has prompted several structure–activity studies on MIF in the search for more active analogues.[18-23] Evaluation of relative activities has mainly been performed by means of an oxotremorine-antagonism test.[18-21] Evidently, the structural requirements for activity are very strict. Only analogues retaining the original tripeptide amide backbone have shown any tremorolytic effect, while tetrapeptides and dipeptides (potential metabolites) related to MIF are inactive.[19] Replacement of the pyrrolidine ring of Pro by a cyclopentane (Cpc) or thiazolidine (Thz) ring causes loss of activity,[21] while substitution of <Glu for Pro even increases the tremorolytic effect.[18]

Modification of the primary amide of Gly generally causes loss of activity,[19-21] the important exception being the highly active <Glu-Leu-Gly-NH-C_2H_5.[19] The lipophilic ethyl group could conceivably facilitate the diffusion of the analogue to its target site, since no mechanism for active uptake of MIF seems to exist.[24] Replacement of Gly-NH_2 by Pro-NH_2 gives some retention of oxotremorine antagonism, while substitution of DL-thiazolidine-2-carboxamide for Gly-NH_2 causes loss of activity in this test but retention of activity in the Dopa potentiation test.[21]

A few analogues have been evaluated as inhibitors of fluphenazine-induced catalepsy.[23] MIF itself was in this test active only after chronic administration, while analogues in which *N*-Me-Leu (D or L) had been substituted for Leu were active after a single injection. In the Dopa potentiation test they were not as potent as MIF.

From the data in the cited literature it is clear that high activity in one test system does not necessarily correlate with high activity in another. Whether any of these systems correlates well with clinical efficiency remains to be established. The picture is further complicated by the observation that very often MIF is active only within a narrow dose range.[25]

The present study explores some possible ways of developing highly active MIF analogues.

Results and Discussion

Chemistry. Peptides **4–8** were prepared from Z-Leu-Gly-OEt (**1**). Acidolysis of **1** and coupling with HCO-Pro[26] (DCI/HOBT) or Cpc-OPcp[27] gave HCO-Pro-Leu-Gly-OEt (**3**) and Cpc-Leu-Gly-OEt (**6**), respectively. Aminolysis with NH_3 or $EtNH_2$ gave the analogues **4**, **5**, **7**, and **8**.

Analogues **13–16**, **20**, **21**, and **25** were prepared from Z-<Glu-Leu (**10**). An attempted direct coupling of Z-<Glu-OPcp and the sodium salt of Leu in dioxane–H_2O (2:1) at pH 11 gave **10** in a 33% yield and 34% of the byproduct Z-Glu-Leu, which accounts for the consumption of 1 equiv of NaOH. Synthesis of **10** by way of the intermediate Z-<Glu-Leu-OBu^t (**9**) was more successful, since the *tert*-butyl ester could be selectively removed with $BF_3 \cdot Et_2O$ in HOAc, giving **10** in an overall yield of 64%. From **10**, <Glu-Leu-Gly-OEt (**12**) was prepared by a DCI/HOBT-mediated condensation with Gly-OEt·HCl

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Table I. Pharmacological Results in Mice

compound	no.	in vivo inhibitory dose[a] mg/kg ± SD	in vivo inhibitory dose[a] μmol/kg ± SD	n^b
Pro-Leu-Gly-NH_2 (MIF)	*c*	36 ± 9.3	127 ± 73	42
HCO-Pro-Leu-Gly-NH_2	4	inact[a]		
HCO-Pro-Leu-Gly-NH-C_2H_5	5	inact[a]		
Cpc-Leu-Gly-NH_2	7	inact[a]		
Cpc-Leu-Gly-NH-C_2H_5	8	inact[a]		
<Glu-Leu-Gly-NH-CH_3	13	inact[a]		
<Glu-Leu-Gly-NH-C_2H_5	*c*	7.8 ± 1.2	24 ± 3.7	36
<Glu-Leu-Gly-NH-C_3H_8	14	10.0 ± 2.1	29 ± 6.2	30
<Glu-Leu-Gly-NH-$CH(CH_3)_2$	15	2.9 ± 0.44	8.5 ± 1.2	66
<Glu-Leu-Gly-NH-$CH_2C{\equiv}CH$	16	inact[a]		
<Glu-Leu-Gly-NH-$C(CH_3)_3$	20	inact[a]		
<Glu-Leu-Gly-$N(CH_3)_2$	21	1.3 ± 0.17	4.0 ± 0.52	36
<Glu-Leu-Gly-$N(CH_3)C_2H_5$	25	inact[a]		
cyclo(-Pro-Leu-)	27	inact[a]		
Pro-Lys-Gly-NH_2·2HOAc	31	inact[a]		
Pro-Arg-Gly-NH_2·2HCl	32	inact[a]		

[a] See Experimental Section for definition. [b] Number of mice used. [c] Included for comparison.[19]

and subsequent hydrogenolysis. From **12**, the amides <Glu-Leu-Gly-NH-R (R = $-CH_3$, $-C_3H_7$, $-i-C_3H_7$, and $-CH_2C{\equiv}CH$) and <Glu-Leu-Gly-$N(CH_3)_2$ (analogues **13–16** and **21**) were prepared by direct aminolysis, although the reaction was slow with the sterically hindered dimethyl- and isopropylamine and with the less nucleophilic propargylamine. <Glu-Leu-Gly-NH-$C(CH_3)_3$ (**20**) and <Glu-Leu-Gly-$N(CH_3)C_2H_5$ (**25**) were prepared from **10** and the corresponding glycine amides. Z-Gly-ONp reacted smoothly with the corresponding sterically hindered amines.

Z-Pro-Leu-OMe (**26**) was synthesized from Z-Pro-ONp and Leu-OMe·HCl. Hydrogenolysis and refluxing in MeOH afforded *cyclo*(-Pro-Leu-) (**27**).

Pro-Lys-Gly-NH_2 (**31**) was prepared by the following route: Boc-Lys(Z) was coupled by means of DCI/HOBT to Gly-NH_2·HCl, giving Boc-Lys(Z)-Gly-NH_2 (**28**). The Boc group was selectively removed by 4 M HCl/dioxane, and the resulting Lys(Z)-Gly-NH_2·HCl (**29**) was coupled with Z-Pro-ONp to yield the protected tripeptide Z-Pro-Lys(Z)-Gly-NH_2 (**30**). Hydrogenolysis in HOAc afforded **31** as a diacetate salt. Pro-Arg-Gly-NH_2 (**32**) was prepared as described in the literature.[28]

Pharmacology. The analogues were tested in vivo as inhibitors of oxotremorine-induced tremor (see Experimental Section for details) and their activities are summarized in Table I.

The observation[18] that substitution of <Glu for Pro in MIF enhanced its activity in this test prompted the investigation of other analogues modified at the amino terminal. The pyrrolidin-4-one ring of the <Glu moiety is only slightly larger than the pyrrolidine ring of Pro. Modifications that further increase the size of the amino terminal moiety cause loss of activity, as evidenced by the inactivity of Thz-Leu-Gly-NH_2,[21] Z-Pro-Leu-Gly-NH_2,[20] and HCO-Pro-Leu-Gly-NH_2 (**4**) with its ethylamide analogue **5**. The lipophilic Cpc-Leu-Gly-NH_2 (**7**) (reported earlier[21]) and its ethylamide analogue **8** were devoid of activity. Thus, the presence of the nitrogen atom of the amino terminal moiety, whether it be basic as in Pro or nonbasic as in <Glu, is essential for activity in this test.

Since no other modification at the amino terminal proved successful, additional <Glu-Leu-Gly-NH_2 analogues were explored. Some of these have very high relative activities; the *n*-propylamide analogue **14**, the isopropylamide **15**, and the dimethylamide analogue **21** are 4, 13, and 29 times as active as MIF. There is no obvious correlation between high activity and high lipophilic character of an analogue. The increased activity of these analogues as compared to MIF is thus probably not simply due to a more effective diffusion into the lipid membranes of the CNS. Generally, only very small alkyl groups are acceptable as amide substituents. The rigid propargyl group and the bulky *tert*-butyl group of the inactive analogues **16** and **20** probably demand too much space. While the dimethylamide **21** proved highly active, the only slightly heavier substituted ethylmethylamide **25** was devoid of activity over a large dosage interval. No clear structure–activity pattern can, however, be discerned as regards the alkylamide analogues, and there remains the possibility that the analogues interact with various slightly different receptors. The existence in the CNS of a number of different receptors sensitive to MIF-like peptides is implied by the diversity of actions of MIF itself and by the findings[21,23] (see Introduction) that a few analogues are active, or even highly active, in one test system and slightly active or inactive in another, while MIF is active in both systems.

MIF is a conformationally flexible molecule, as has been demonstrated by means of ^{13}C NMR.[29] The simplest rigid analogue containing most of the structural elements of MIF is *cyclo*(-Pro-Leu-) (**27**), an analogue that should also be less sensitive to enzymatic attack. This structural modification proved, however, too large to permit retention of biological activity.

Pro-Leu-Gly-NH_2 is the C-terminal tripeptide of oxytocin. The corresponding fragments of porcine and bovine vasopressin are Pro-Lys-Gly-NH_2 (**31**) and Pro-Arg-Gly-NH_2 (**32**). In a MIF bioassay (inhibition of MSH release from rat pituitary gland), they are both active, although less potent than MIF.[30] In our oxotremorine antagonism test they had no effect. Conversely, <Glu^1-MIF had no effect in the MIF bioassay.[30] Apparently, the structural requirements for activity are different for MSH release-inhibiting effect and CNS effects.

We also tested MIF and one analogue (<Glu-Leu-Gly-NH-C_2H_5) as antagonists of fluphenazine-induced catalepsy in rats (male Sprague–Dawley, 220–260 g) under the experimental conditions described by Voith.[23] In our rat strain no such antagonism was observed.[31]

In conclusion, small modifications of the structure of MIF can yield highly active analogues with potential clinical value, e.g., in the treatment of Parkinson's disease or mental depression.

Experimental Section

Melting points were determined with an electrically heated

metal block, using calibrated Anschütz thermometers. Microanalyses were performed in the analytical laboratory of W. Kirsten, University of Agriculture, Uppsala. When analyses are indicated by the symbols of the elements, analytical results were within ±0.4% of the theoretical values. NMR spectra were obtained for all compounds, and the expected signals were identified. The optical rotations were measured on a Perkin-Elmer Model 141 or 241 readout polarimeter using a microcell. All reactions were monitored by TLC, and the purity of all products was checked in three different TLC systems. R_f^1, R_f^2, and R_f^3 refer to TLC on silica gel (Merck F 254 precoated plates) in HOAc–EtOAc–BuOH–H_2O (1:1:1:1), EtOH–H_2O–EtOAc (7:4:8), and $CHCl_3$–MeOH–NH_3 (concentrated) (50:20:5), respectively (R_f^1 0.55, R_f^2 0.45, and R_f^3 0.20 for Phe).

Evaporations of solvent were always in vacuo at a temperature not exceeding 40 °C. Chloroform for the column chromatographies was commercial Merck GR grade, and the anhydrous DMF for coupling reactions was freshly distilled from ninhydrin under reduced pressure. All amino acids except Gly were purchased as the pure L isomers. The symbols of the amino acids and peptides follow the IUPAC–IUB recommendations.[32] The following abbreviations are used: Pcp, pentachlorophenyl; DCI, dicyclohexylcarbodiimide; HOBT, 1-hydroxybenzotriazole; Cpc, cyclopentanecarboxylic acid.

***N*-(Benzyloxycarbonyl)leucylglycine Ethyl Ester (1).** Z-Leu (3.98 g, 15 mmol) and HOBT (2.23 g, 16.5 mmol) were dissolved in DMF (25 mL). The solution was chilled with ice, and DCI (3.09 g, 15 mmol) was added. After stirring the solution for 1 h, Gly-OEt·HCl (2.09 g, 15 mmol) and Et_3N (2.10 mL, 15 mmol) were added and the ice bath was removed. The mixture was stirred for 6 h at room temperature, chilled overnight, and filtered. The solvent was evaporated, and the residue was partitioned between 5% $NaHCO_3$ solution and EtOAc (2 × 30 mL of each). The organic phase was dried over Na_2SO_4, the solvent was evaporated, and the residue was crystallized from EtOH–H_2O to yield pure 1: yield 3.94 g (75%); mp 100.5–102 °C; $[\alpha]^{22}_D$ −27.0° (*c* 5, EtOH) [lit.[33] mp 104–105 °C; $[\alpha]^{22}_D$ −27.1° (*c* 5, EtOH)]; TLC R_f^1 0.82, R_f^2 0.88, R_f^3 0.96. Anal. ($C_{18}H_{26}N_2O_5$) C, H, N.

***N*-Formylprolylleucylglycine Ethyl Ester (3).** Compound 1 (1.40 g, 4 mmol) was dissolved in 3.5 M HBr/HOAc (10 mL). The solution was stirred for 40 min, and the obtained Leu-Gly-OEt·HBr (2) was precipitated with Et_2O (80 mL). Drying for 24 h in vacuo over KOH brought its weight down to 1.20 g (101%): TLC R_f^1 0.63, R_f^2 0.55, R_f^3 0.87. This was coupled with HCO-Pro (0.572 g, 4 mmol) in DMF (10 mL) by means of DCI (0.824 g, 4 mmol), HOBT (0.675 g, 5 mmol), and Et_3N (0.56 mL, 4 mmol) as described for 1. After the evaporation of the DMF, the residue was dissolved in EtOAc (30 mL) and extracted with 5% $NaHCO_3$ solution (2 × 30 mL). The combined aqueous phases were washed several times with EtOAc, the combined organic phases were dried over Na_2SO_4, and the solvent was evaporated. The crude product was purified on a silica column eluted with a MeOH–$CHCl_3$ gradient starting with pure $CHCl_3$. MeOH–$CHCl_3$ (1%) eluted the pure 3 as a hygroscopic oil: yield 0.876 g (64%); TLC R_f^1 0.63, R_f^2 0.73, R_f^3 0.90. Anal. ($C_{16}H_{27}N_3O_5$) C, H, N.

***N*-Formylprolylleucylglycine Amide (4).** Compound 3 (0.205 g, 0.6 mmol) was dissolved in MeOH saturated (in the cold) with NH_3 (5 mL). After 3 h the solvent was evaporated. The residue was dissolved in H_2O and lyophilized to yield pure 4: yield 0.191 g (100%); no sharp mp; $[\alpha]^{22}_D$ −97.4° (*c* 1, MeOH); TLC R_f^1 0.49, R_f^2 0.55, R_f^3 0.62. Anal. ($C_{14}H_{24}N_4O_4$) C, H, N.

***N*-Formylprolylleucylglycine Ethylamide (5).** Compound 3 (0.478 g, 1.4 mmol) was treated with $EtNH_2$ (2 mL) in MeOH (4 mL) for 6 h. Evaporation of the solvent and crystallization from MeOH–Et_2O gave pure 5: yield 0.431 g (90%); no sharp mp; $[\alpha]^{22}_D$ −76.7° (*c* 1, MeOH); TLC R_f^1 0.55, R_f^2 0.61, R_f^3 0.84. Anal. ($C_{16}H_{28}N_4O_4 \cdot 0.5\ H_2O$) C, H, N.

***N*-(Cyclopentylcarbonyl)leucylglycine Ethyl Ester (6).** Compound 1 (2.10 g, 6 mmol) was treated as described for 3, giving 2 (1.79 g, 100%). This was dissolved in DMF (10 mL) and added to a solution of Cpc-OPcp (2.18 g, 6 mmol) in CH_2Cl_2 (2.5 mL). After the addition of Et_3N (0.84 mL, 6 mmol), the reaction mixture was stirred for 3 h, and after the addition of more Et_3N (0.31 mL, 2 mmol) for another 6 h. The solvent was evaporated and the residue was purified on a silica column eluted with $CHCl_3$. Trituration of the crude product with heptane and crystallization of the solid residue from EtOH–H_2O gave pure 6: yield 0.98 g (53%); mp 119–121 °C; $[\alpha]^{22}_D$ −47.2° (*c* 1, MeOH); TLC R_f^1 0.80, R_f^2 0.86, R_f^3 0.96. Anal. ($C_{16}H_{28}N_2O_4$) C, H, N.

***N*-(Cyclopentylcarbonyl)leucylglycine Amide (7).** Compound 6 (0.187 g, 0.6 mmol) was dissolved in MeOH saturated (in the cold) with NH_3 (20 mL). After 6 h, the solvent was evaporated and the residue crystallized from EtOH–H_2O, giving pure 7: yield 0.150 g (88%); mp 172–173 °C; $[\alpha]^{22}_D$ −19.9° (*c* 1, MeOH) [lit.[21] mp 170–172 °C; $[\alpha]^{24}_D$ −20.6° (*c* 1.0, MeOH)]; TLC R_f^1 0.72, R_f^2 0.79, R_f^3 0.75. Anal. ($C_{14}H_{25}N_3O_3$) C, H; N: calcd, 14.8; found, 14.3.

***N*-(Cyclopentylcarbonyl)leucylglycine Ethylamide (8).** Compound 6 (0.156 g, 0.5 mmol) was dissolved in $EtNH_2$ (5 mL), and the mixture was stirred for 8 h. Evaporation of the amine and crystallization of the residue from EtOH–H_2O gave pure 8: yield 0.145 g (93%); mp 168–169.5 °C; $[\alpha]^{22}_D$ +0.5° (*c* 3, MeOH); TLC R_f^1 0.78, R_f^2 0.84, R_f^3 0.89. Anal. ($C_{16}H_{29}N_3O_3$) C, H, N.

***N*-(Benzyloxycarbonyl)pyroglutamylleucine *tert*-Butyl Ester (9).** Z-<Glu (2.11 g, 8 mmol) and Leu-OBu^t·HCl (1.92 g, 8 mmol) were coupled in DMF (15 mL) by means of DCI (1.65 g, 8 mmol), HOBT (1.22 g, 9 mmol), and Et_3N (1.1 mL, 8 mmol) as described for 1, and the workup was similar. Crystallization from EtOAc–heptane yielded pure 9: yield 2.68 g (78%); mp 120–121 °C; $[\alpha]^{22}_D$ −73.4° (*c* 1, MeOH); TLC R_f^1 0.87, R_f^2 0.88, R_f^3 0.94. Anal. ($C_{23}H_{32}N_2O_6$) C, H, N.

***N*-(Benzyloxycarbonyl)pyroglutamylleucine (10).** $BF_3 \cdot Et_2O$ (0.35 mL, ca. 2 mmol) was added to a solution of 9 (0.43 g, 1 mmol) in glacial HOAc (5 mL), and the mixture was stirred under N_2 for 1 h. TLC indicated >90% reaction. Another 0.1 mL of $BF_3 \cdot Et_2O$ was added, and the stirring continued for 1 h. The solvent was evaporated and the product was precipitated by the addition of H_2O. Crystallization from H_2O gave pure 10: yield 0.309 g (82%); mp 186–188 °C; $[\alpha]^{22}_D$ −57.6° (*c* 1, MeOH). Anal. ($C_{19}H_{24}N_2O_6$) C, H, N. 10 obtained in a 33% yield by the coupling of Z-<Glu-OPcp and Leu·Na in dioxane–H_2O (2:1) at pH 11 had mp 187–189 °C; $[\alpha]^{22}_D$ −51.4° (*c* 1, MeOH); the two products were identical on TLC, R_f^1 0.76, R_f^2 0.57, R_f^3 0.17.

***N*-(Benzyloxycarbonyl)pyroglutamylleucylglycine Ethyl Ester (11).** Compound 10 (3.01 g, 8 mmol) and Gly-OEt·HCl (1.12 g, 8 mmol) were coupled in DMF (20 mL) by means of DCI (1.65 g, 8 mmol), HOBT (1.44 g, 11 mmol), and Et_3N (1.11 mL, 8 mmol) as described for 1. Similar workup and crystallization from 96% EtOH gave pure 11: yield 3.12 g (85%); mp 147–149 °C; $[\alpha]^{22}_D$ −83.4° (*c* 1, MeOH); TLC R_f^1 0.70, R_f^2 0.83, R_f^3 0.94. Anal. ($C_{23}H_{31}N_3O_7$) C, H, N.

Pyroglutamylleucylglycine Ethyl Ester (12). Compound 11 (1.85 g, 4 mmol) was hydrogenolyzed for 3 h in MeOH at atmospheric pressure and room temperature using 10% Pd on $BaSO_4$ as catalyst. Filtration of the solution, evaporation of the solvent, and crystallization from EtOAc–heptane gave pure 12: yield 1.24 g (95%); no sharp mp; the product was identical with a reference sample on TLC.[19]

Pyroglutamylleucylglycine Alkylamides. Compound 12 (0.5 mmol) was dissolved in the appropriate amine (if necessary by the addition of EtOH) and the mixture was stirred until the reaction was complete by TLC. Products 13–15 could be directly crystallized from MeOH–Et_2O, while 16 and 21 were first purified by preparative TLC (15% MeOH–$CHCl_3$).

Methylamide 13: reaction time 2 h; yield 95%; mp 176–178 °C; $[\alpha]^{22}_D$ −3.9° (*c* 1, MeOH); TLC R_f^1 0.49, R_f^2 0.60, R_f^3 0.71. Anal. ($C_{14}H_{24}N_4O_4$) C, H, N.

Propylamide 14: reaction time 6 h; yield 85%; mp 179–180 °C; $[\alpha]^{22}_D$ +4.4° (*c* 1, MeOH); TLC R_f^1 0.60, R_f^2 0.71, R_f^3 0.79. Anal. ($C_{16}H_{28}N_4O_4$) C, H, N.

Isopropylamide 15: reaction time 5 days; yield 85%; mp 208–210 °C; $[\alpha]^{22}_D$ +10.7° (*c* 1, MeOH); TLC R_f^1 0.60, R_f^2 0.70, R_f^3 0.80. Anal. ($C_{16}H_{28}N_4O_4$) C, H, N.

Propargylamide 16: reaction time 10 days; yield 64%; mp 166–167 °C; $[\alpha]^{22}_D$ −4.0° (*c* 1, MeOH); TLC R_f^1 0.72, R_f^2 0.73, R_f^3 0.72. Anal. ($C_{16}H_{24}N_4O_4$) C, H, N.

Dimethylamide 21: reaction time 2 days; yield 62%; mp 137–139 °C; $[\alpha]^{22}_D$ −53.0° (*c* 1, MeOH). TLC R_f^1 0.46, R_f^2 0.51, R_f^3 0.84. Anal. ($C_{15}H_{26}N_4O_4$) C, H, N.

***N*-(Benzyloxycarbonyl)glycine *tert*-Butylamide (17).**

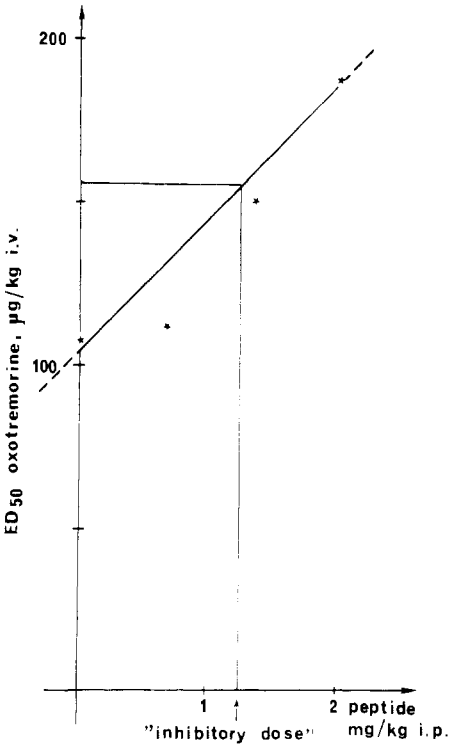

Figure 1. The regression line of analogue **21**. Two groups of six mice were used at the highest point and at the zero point.

Z-Gly-ONp (1.32 g, 4 mmol) was dissolved in DMF (7 mL) and *t*-BuNH$_2$ (7 mL) was added. The mixture was stirred overnight, the solvent was evaporated, and the product was isolated by means of a Lobar® Si-60 size B column eluted with $CHCl_3$. Crystallization from MeOH–H_2O gave pure **17**: yield 0.330 g (31%); mp 68.5–71 °C; TLC R_f^1 0.89, R_f^2 0.88, R_f^3 0.92. Anal. ($C_{14}H_{20}N_2O_3$) C, H, N.

***N*-(Benzyloxycarbonyl)pyroglutamylleucylglycine *tert*-Butylamide (19).** Compound **17** (0.290 g, 1.1 mmol) was hydrogenolyzed as described for **12**, giving Gly-NH-Bu^t (**18**) (0.154 g of crude product) as a pale yellow oil: TLC R_f^1 0.51, R_f^2 0.16 + 0.26 (possibly rotational isomers, see below), R_f^3 0.82 (positive to ninhydrin reagent). This was coupled with **10** (0.414 g, 1.1 mmol) by means of DCI (0.227 g, 1.1 mmol) and HOBT (0.176 g, 1.3 mmol) in DMF (5.5 mL) as described for **1**. After the evaporation of the DMF, the product was isolated by means of a Lobar® Si-60 size B column eluted with a MeOH–$CHCl_3$ gradient starting with pure $CHCl_3$. MeOH–$CHCl_3$ (2%) eluted the product in two peaks (0.096 and 0.347 g), with identical NMR and mass spectra, possibly corresponding to the two rotational isomers of the *tert*-butylamide. TLC gave R_f^1 0.83, R_f^2 0.86, and R_f^3 0.89 for both isomers. Crystallization of the main fraction from MeOH gave pure **19**: yield 0.293 g (55%); mp 191–193 °C; $[\alpha]^{22}_D$ −47.4° (*c* 1, MeOH). Anal. ($C_{25}H_{36}N_4O_6$) C, H, N.

Pyroglutamylleucylglycine *tert*-Butylamide (20). Compound **19** (0.195 g, 0.4 mmol) was hydrogenolyzed as described for **12**. Crystallization from MeOH gave pure **20**: yield 0.085 g (60%); mp 221–223.5 °C; $[\alpha]^{22}_D$ −12.3° (*c* 1, MeOH); TLC R_f^1 0.78, R_f^2 0.76, R_f^3 0.80. Anal. ($C_{17}H_{30}N_4O_4$) C, H; N: calcd, 15.8; found, 15.2.

***N*-(Benzyloxycarbonyl)glycine Ethylmethylamide (22).** EtNHMe (0.32 mL) was added to Z-Gly-ONp (0.40 g, 1.2 mmol) during ice cooling. The reaction was immediate. The product was purified on a Hibar® Si-60 column eluted with $CHCl_3$. Crystallization from MeOH–H_2O gave pure **22**: yield 0.24 g (80%); mp 53.5–55 °C; TLC R_f^1 0.80, R_f^2 0.86, R_f^3 0.92. Anal. ($C_{13}H_{18}N_2O_3$) C, H, N.

***N*-(Benzyloxycarbonyl)pyroglutamylleucylglycine Ethylmethylamide (24).** Compound **22** (0.230 g, 0.92 mmol) was hydrogenolyzed as described for **12**. Gly-N(CH_3)C_2H_5 (**23**; 0.104 g, 97%) was obtained as a colorless oil: TLC R_f^1 0.44, R_f^2 0.14, R_f^3 0.78 (positive to ninhydrin reagent). This was coupled with **10** (0.376 g, 1 mmol) by means of DCI (0.206 g, 1 mmol) and HOBT (0.149 g, 1.1 mmol) in DMF (5 mL) as described for **1**. After the evaporation of the DMF, the product was isolated by means of a Lobar® Si-60 size B column eluted with a MeOH–$CHCl_3$ gradient starting with pure $CHCl_3$–MeOH–$CHCl_3$ (3%). Crystallization from EtOH–H_2O yielded pure **24**: yield 0.353 g (83%); mp 156.5–158 °C; $[\alpha]^{22}_D$ −90.9° (*c* 1, MeOH); TLC R_f^1 0.72, R_f^2 0.73, R_f^3 0.93. Anal. ($C_{24}H_{34}N_4O_6$) C, H, N.

Pyroglutamylleucylglycine Ethylmethylamide (25). Compound **24** (0.190 g, 0.4 mmol) was hydrogenolyzed as described for **12**. Crystallization from MeOH–Et_2O gave pure **25**: yield 0.124 g (91%); mp 129–131 °C; $[\alpha]^{22}_D$ −54.1° (*c* 1, MeOH); TLC R_f^1 0.64, R_f^2 0.60, R_f^3 0.84. Anal. ($C_{16}H_{28}N_4O_4$) C, H, N.

***N*-(Benzyloxycarbonyl)prolylleucine Methyl Ester (26).** Z-Pro-ONp (1.85 g, 5 mmol) and Leu-OMe·HCl (0.91 g, 5 mmol) were dissolved in DMF (7 mL). Et_3N was added in portions (1.27 mL, 9 mmol in all) during the 8-h stirring. The solvent was evaporated and the residue dissolved in EtOAc (50 mL). The solution was washed with 5% $NaHCO_3$ solution (14 × 20 mL) and dried over Na_2SO_4, and the solvent was evaporated. Crystallization from MeOH–H_2O and thorough washing with H_2O gave pure **26**: yield 1.59 g (84%); mp 76.5–78 °C; $[\alpha]^{22}_D$ −78.5° (*c* 1, MeOH); TLC R_f^1 0.76, R_f^2 0.87, R_f^3 0.95. Anal. ($C_{20}H_{28}N_2O_5$) C, H, N.

***cyclo*-(Prolylleucyl) (27).** Compound **26** (0.264 g, 0.7 mmol) was hydrogenolyzed as described for **12**. The filtered solution was refluxed for 10 h. Evaporation of the solvent and crystallization from EtOAc gave pure **27**: yield 0.081 g (55%); mp 160–163 °C; $[\alpha]^{22}_D$ −134° (*c* 1, MeOH) [lit.[34] mp 157–158 °C; $[\alpha]^{22}_D$ −133° (*c* 1, EtOH)]; TLC R_f^1 0.68, R_f^2 0.72, R_f^3 0.92. Anal. ($C_{11}H_{18}N_2O_2$) C, H, N.

***N*$^\alpha$-(*tert*-Butyloxycarbonyl)-*N*$^\epsilon$-(benzyloxycarbonyl)-lysylglycine Amide (28).** Boc-Lys(Z) (2.90 g, 7.6 mmol) and Gly-NH_2·HCl (0.84 g, 7.6 mmol) were coupled in DMF (20 mL) by means of DCI (1.57 g, 7.6 mmol), HOBT (1.10 g, 8.1 mmol), and Et_3N (1.06 mL, 7.6 mmol) as described for **1**. After evaporation of the DMF, the product was isolated by means of an Al_2O_3 column (100 g) eluted with $CHCl_3$. Crystallization from EtOAc–heptane yielded pure **28**: yield 2.96 g (89%); mp 68–70 °C; $[\alpha]^{22}_D$ −3.1° (*c* 1, MeOH); TLC R_f^1 0.86, R_f^2 0.87, R_f^3 0.78. Anal. ($C_{21}H_{32}N_4O_6$) C, H, N.

***N*-(Benzyloxycarbonyl)prolyl-*N*$^\epsilon$-(benzyloxycarbonyl)-lysylglycine Amide (30).** Compound **28** (0.784 g, 1.8 mmol) was treated with 4.1 M HCl/dioxane (15 mL) for 2 h at room temperature. The product was precipitated by the addition of Et_2O and dried overnight in vacuo over KOH. This gave >95% pure Lys(Z)-Gly-NH_2·HCl (**29**): yield 0.616 g (92%); TLC R_f^1 0.52, R_f^2 0.31, R_f^3 0.51 (positive to ninhydrin reagent). Compound **29** (0.60 g, 1.6 mmol) and Z-Pro-ONp (0.71 g, 1.9 mmol) were dissolved in DMF (15 mL), and Et_3N (0.22 mL, 1.6 mmol) was added. The mixture was stirred for 18 h, the solvent was evaporated, and the product was isolated on a silica column eluted with a MeOH–$CHCl_3$ gradient starting with pure $CHCl_3$. MeOH–$CHCl_3$ (6%) eluted the product. Crystallization from EtOH–heptane gave pure **30**: yield 0.723 g (80%); mp 142–148 °C dec; $[\alpha]^{22}_D$ −43.6° (*c* 1, MeOH); TLC R_f^1 0.69, R_f^2 0.76, R_f^3 0.85. Anal. ($C_{29}H_{37}N_5O_7$) C, H, N.

Prolyllysylglycine Amide Diacetate (31). Compound **30** (0.170 g, 0.3 mmol) was hydrogenolyzed in HOAc (10 mL) as described for **12**. The product was lyophilized four times from H_2O to remove excess HOAc. This gave pure **31**: yield 0.112 g (89%); no sharp mp; $[\alpha]^{22}_D$ −36.9° (*c* 1.3, H_2O) [lit.[35] Pro-Lys-Gly-NH_2·2HCl·0.5H_2O: $[\alpha]^{22}_D$ −40.0° (*c* 1.8, H_2O)]; TLC R_f^1 0.15, R_f^2 0.02, R_f^3 0.15. Anal. ($C_{13}H_{25}N_5O_3$·2HOAc·1.5H_2O) C, H, N.

Oxotremorine Antagonism Test. A full description of the method has been given.[25] The oxotremorine-induced tremor was measured by means of an electronic transducer.[36] Oxotremorine was administered to groups of six male NMRI mice weighing 20–26 g, and the median dose (ED_{50}) required to evoke a predetermined tremor intensity was calculated. To other groups, various doses (ranging from 0.13 to 16 mg/kg) of peptide were given intraperitoneally 1 h before the administration of oxotremorine. The median effective dose of oxotremorine was plotted against the dose of the tested peptide. An analogue was considered active if a linear regression (Figure 1) could be calculated[37] on at least four doses, including zero. The activity of an analogue is expressed as the dose of peptide after which 50% more oxotremorine would be required to evoke the predetermined tremor response. This is referred to as the "inhibitory dose". At doses equal to or higher

than twice the inhibitory dose the analogues were always inactive. This holds true also for MIF itself.[25]

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References and Notes

(1) N. P. Plotnikoff, A. J. Kastin, M. S., Anderson, and A. V. Schally, *Life Sci.*, **10**, 1279 (1971).
(2) J. P. Huidobro-Toro, A. Scotti de Carolis, and V. G. Longo, *Pharmacol. Biochem. Behav.*, **3**, 235 (1975).
(3) N. P. Plotnikoff and A. J. Kastin, *Arch. Int. Pharmacodyn.*, 211, 211 (1974).
(4) N. P. Plotnikoff, A. J. Kastin, M. S. Anderson, and A. V. Schally, *Proc. Soc. Exp. Biol. Med.*, **140**, 811 (1972).
(5) N. P. Plotnikoff and A. J. Kastin, *Pharmacol. Biochem. Behav.*, **2**, 417 (1974).
(6) N. P. Plotnikoff, A. J. Kastin, M. S. Anderson, and A. V. Schally, *Neuroendocrinology*, 11, 67 (1973).
(7) R. Walter, P. L. Hoffman, J. B. Flexner, and L. B. Flexner, *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 4180 (1975).
(8) J. M. van Ree and D. de Wied, *Life Sci.*, **19**, 1331 (1976).
(9) R. B. North, S. I. Harik, and S. H. Snyder, *Brain Res.*, **63**, 435 (1973).
(10) L. O. Stratton and A. J. Kastin, *Pharmacol. Biochem. Behav.*, **3**, 901 (1975).
(11) T. J. Crowley and M. Hydinger, *Pharmacol. Biochem. Behav.*, **5** (Suppl 1), 79 (1976).
(12) A. J. Kastin and A. Barbeau, *Can. Med. Assoc. J.*, **107**, 1079 (1972).
(13) F. Gerstenbrand, H. Binder, C. Kozma, S. Pusch, and T. Reisner, *Wien. Klin. Wochenschr.*, **87**, 822 (1975).
(14) A. Barbeau, M. Roy, and A. J. Kastin, *Can. Med. Assoc. J.* **114**, 120 (1976).
(15) R. H. Ehrensing and A. J. Kastin, *Arch. Gen. Psychiatry*, **30**, 63 (1974).
(16) R. H. Ehrensing and A. J. Kastin, *Pharmacol. Biochem. Behav.*, **5** (Suppl 1), 89 (1976).
(17) R. H. Ehrensing and A. J. Kastin, *Am. J. Psychiatry*, **135**, 562 (1978).
(18) S. Castensson, H. Sievertsson, B. Lindeke, and C. Y. Sum, *FEBS Lett.*, **44**, 101 (1974).
(19) S. Björkman, S. Castensson, B. Lindeke, and H. Sievertsson, *Acta Pharm. Suec.*, **13**, 289 (1976).
(20) H. Sievertsson, S. Castensson, S. Björkman, and C. Y. Bowers in "Hypothalamic Hormones—Chemistry, Physiology, and Clinical Applications", D. Gupta and W. Voelter, Eds., Verlag Chemie GmbH, Weinheim, West Germany, 1978, p 145.
(21) R. L. Johnson, E. E. Smissman, and N. P. Plotnikoff, *J. Med. Chem.*, **21**, 165 (1978).
(22) A. Failli, K. Sestanj, H. U. Immer, and M. Götz, *Arzneim.-Forsch.*, **27**(II), 2286 (1977).
(23) K. Voith, *Arzneim.-Forsch.*, **27**(II), 2290 (1977).
(24) R. Greenberg, C. E. Whalley, F. Jourdikian, I. S. Mendelson, R. Walter, K. Nicolics, D. H. Coy, A. V. Schally, and A. J. Kastin, *Pharmacol. Biochem. Behav.*, **5** (Suppl 1), 151 (1976).
(25) S. Björkman and H. Sievertsson, *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **298**, 79 (1977).
(26) M. T. Pizzorno and S. M. Albonico, *J. Org. Chem.*, **39**, 731 (1974).
(27) H. Sievertsson, S. Castensson, K. Andersson, S. Björkman, and C. Y. Bowers, *Biochem. Biophys. Res. Commun.*, **66**, 1401 (1975).
(28) R. O. Studer and V. du Vigneaud, *J. Am. Chem. Soc.*, **82** 1499 (1960).
(29) R. Deslauriers, R. Walter, and I. C. P. Smith, *FEBS Lett.*, **37**, 27 (1973).
(30) M. E. Celis, S. Hase, and R. Walter, *FEBS Lett.*, **27**, 327 (1972).
(31) S. Björkman and T. Lewander, unpublished results.
(32) IUPAC–IUB Commission on Biochemical Nomenclature, *Biochem. J.*, **126**, 773 (1972).
(33) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).
(34) D. E. Nitecki, B. Halpern, and J. W. Westley, *J. Org. Chem.*, **33**, 864 (1968).
(35) S. Sakakibara, T. Fukuda, Y. Kishida, and I. Honda, *Bull. Chem. Soc. Jpn.*, **43**, 3322 (1970).
(36) R. W. Silverman and D. J. Jenden, *J. Appl. Physiol.*, **28**, 513 (1970).
(37) G. W. Snedecor "Statistical Methods", 5th ed, The Iowa State College Press, Ames, Iowa, 1959.

Synthesis and Biological Activity of Peptide Antagonists of Luliberin (Luteinizing Hormone-Releasing Hormone)

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A series of des-His[2] octa- and nonapeptide analogues of luliberin (luteinizing hormone-releasing hormone) with modifications in the 1 and 6 positions, and in some instances the 10 position, has been prepared. Some of these analogues are potent inhibitors of luliberin in vitro and in vivo. The use of ultraviolet absorption measurements for evaluating peptides containing tyrosine and tryptophan is described. An efficient synthesis of *O*-methyl-D-tyrosine is reported.

The first analogue reported to be an antagonist of luliberin [luteinizing hormone-releasing hormone (LH-RH)] was [des-His[2]]-LH-RH, described by Vale et al.[4] Since that time, a large number of antagonists to luliberin have been synthesized and tested. The most potent analogues have been modified at the 2, 3, 6, and 10 positions of LH-RH. A review by Schally et al.[5] summarizes the literature in this field. Recent reports from Folkers' group[6-8] and Schally's group[9] describe additional analogues based on this approach.

Luliberin agonist analogues in which the 1 position has been modified have been reported.[10-12] However, only a few reports have appeared in which antagonists of luliberin have been obtained by modification of the 1 position. Bowers et al.[13] reported that replacement of <Glu[1] with the nitrogen mustard chlorambucil (Chl) in [Chl[1],Leu[2],-

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