The HCl salt melted at 216° (EtOH). *Anal.* ($C_{10}H_{15}N_3O \cdot HCl$) C, H, N, Cl.

1-[4-(2,6-Dimethoxypyrimidinyl)]piperazine was prepd similarly in 50% yield, light yellow oil, bp 165° (0.1 mm), $n^{25}D$ 1.5560. The HCl salt melted at 205–207° (MeOH). *Anal.* ($C_{10}H_{16}N_4O_2 \cdot HCl$), C, H, N.

1-[4-(4-Methylpyrimidinyl)]- and 1-[4-(4,6-dimethylpyrimidinyl)]-piperazines were prepd according to Janssen.[6]

1-(ω-Cyonoalkyl)-4-pyrimidinyl piperazines were obtd by condensing equimolar amounts of ω-chloroalkylnitriles and appropriate 1-pyrimidinyl piperazines with excess anhyd Na_2CO_3 in *n*-BuOH in 68–94% yields. The physical constants of new compds are listed in Table IV.

1-(ω-Aminoalkyl)-4-pyridylpiperazines were obtd according to Mull, *et al.*[7]

1-(ω-Aminoalkyl)-4-pyrimidinylpiperazines were prepd from the corresponding ω-cyanoalkyl derivatives either by a LAH reduction or a catalytic hydrogenation at room temp under 84 kg/cm² pressure of H_2 with W-6 Raney Ni catalyst. These compds were very hygroscopic and not analyzed. They were purified by distn under reduced pressure and used immediately for the next reaction.

8-(4-Substituted 1-Piperazinylalkyl)-8-azaspiro[4.5]decane-7,9-diones. Method A. A mixt of 8-(ω-chloroalkyl)-8-azaspiro[4.5]-decane-7,9-dione (0.1 mole), 1-substituted piperazine (0.1 mole), Na_2CO_3 (0.1 mole), and *n*-BuOH was refluxed for 15 hr, and filtered. The filtrate was concd and distd to give the product. Method B. An equimolar mixt of 3,3-tetramethyleneglutaric anhydride and 1-(ω-aminoalkyl)piperazine in dry pyridine (0.1 mole/400 ml) was refluxed for 15 hr. It was concd; if the ir spectrum showed typical imide bands (1700 and 1710 cm^{-1}), the residue was purified by either distn or crystn. If the spectrum showed amide acid bands (1680, 1760, 330 cm^{-1}) instead, the residue was refluxed with 10 times its wt of Ac_2O for 15 hr. The residue obtd by removal of Ac_2O was purified either by distn or recrystn. The HCl salts were prepd by treating the free bases with an equiv amt of ethanolic HCl.

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Synthetic Thyrotropin-Releasing Factor Analogs. 3.[1,2] Effect of Replacement or Modification of Histidine Residue on Biological Activity

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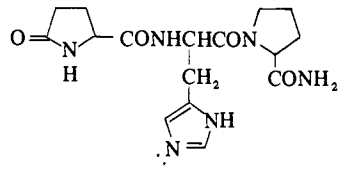
A series of analogs of TRF (thyrotropin-releasing factor, pGlu-His-Pro-NH_2) in which His was replaced by a basic, aromatic, or S-containing amino acid was synthesized and tested for TRF biological activity. pGlu-3-Me-His-Pro-NH_2 appeared to be 10 times more active biologically than the natural or synthetic TRF itself while pGlu-1-Me-His-Pro-NH_2 was almost completely inactive. [Orn^2]-, [Lys^2]-, [Arg^2]-, [Tyr^2]-TRF showed less than 0.1% of TRF activity while [Met^2]-TRF had 1% of TRF activity. All the activities were measured *in vivo*. Merrifield's solid-phase synthesis on a benzhydrylamine resin was applied for the preparation of all the compounds. pGlu-1-Me-His-Pro-NH_2 and pGlu-3-Me-His-Pro-NH_2 were also synthesized by a classical method. The final products, homogeneous in 4 different tlc systems, were characterized by means of amino acid analysis, nmr, and mass spectrometry.

A peptide molecule as structurally simple as TRF (thyrotropin-releasing factor: pGlu-His-Pro-NH_2 (**1**)), with such



TRF

high specific biological activity as well as such high specificity of action, is a model of choice for the synthesis of a series of analogs which would allow the study of relationships between specific changes in the molecular structure and modification of biological activity. Many such derivatives have already been described by Burgus,[1] Hofmann,[3a] Bowers,[3b] Chang,[3c] Nicolaides,[3d] Wilber,[3e] Gillessen,† and Vale[2] in which each of the 3 amino acid residues of the TRF molecule have been replaced or modified.

†Gillessen, *et al.*,[3f] reported the synthesis of [Lys^2]-TRF, [α,γ-diaminobutyryl^2]-TRF, [β-(3-pyrazolyl)alanine^2]-TRF, and [arginine^2]-TRF and concluded that their compounds exhibited the characteristic biological responses of TRF.

Among the analogs which have been described so far, all of the alterations of structure have resulted in a drastic reduction of specific biological activity. We now describe the synthesis of a series of 6 new tripeptides with changes of the imidazole group of histidine and substitution of histidine by a basic, aromatic, and S-containing L-amino acid; the biological assay data on these latter compounds are included.

Synthesis. The approach for the classical synthesis of pyroglutamyl-1-Me-histidylprolinamide (**1a**) and pyroglutamyl-3-Me-histidylprolinamide (**1b**) was essentially that described by Gillessen, *et al.*[4] (Scheme I). However the presence of Me acting as a protective group on the imidazole gave much better yields. *N*-Carbobenzoxypyroglutamic acid[5] (**4**) and 1-methylhistidine methyl ester (**2a**) or 3-methylhistidine methyl ester (**2b**) were first coupled by *N,N*-dicyclohexylcarbodiimide (DCI), affording the resulting dipeptides *N*-carbobenzoxypyroglutamyl-1-methylhistidine methyl ester (**3a**) and *N*-carbobenzoxypyroglutamyl-3-methylhistidine methyl ester (**3b**), respectively. Hydrogenolysis (Pd/C) yielded the 2 dipeptides pyroglutamyl-1-methylhistidine methyl ester (**5a**) and pyroglutamyl-3-methylhistidine methyl ester (**5b**), which upon treatment with hydrazine in

Scheme I

Z-pGlu + 1- or 3-CH_3-His-OCH_3 $\xrightarrow{DCI}$ Z-pGlu-1- or 3-CH_3-His-OCH_3
4 2a or 2b 3a or 3b

$\xrightarrow{H_2, Pd/C}$ pGlu-1- or 3-CH_3-His-OCH_3
5a or 5b

$\xrightarrow{H_2NNH_2}$ p-Glu-1- or 3-CH_3-His-NH-NH_2
6a or 6b

$\xrightarrow[Pro\text{-}NH_2]{\text{azide coupling}}$ p-Glu-1- or 3-CH_3-His-Pro-NH_2 ≡[1- or 3-CH_3-His²]-TRF
1a or 1b

MeOH yielded the corresponding hydrazides: pyroglutamyl-1-methylhistidine hydrazide (**6a**) and pyroglutamyl-3-methylhistidine hydrazide (**6b**) in very good yields. Coupling with prolinamide was performed after conversion of the hydrazides **6a** and **6b** to their respective azides by HNO_2 treatment. The final products, pyroglutamyl-1-methylhistidylprolinamide (**1a**) ([1-Me-His²]-TRF)‡ and pyroglutamyl-3-methylhistidylprolinamide (**1b**) ([3-Me-His²]-TRF) were purified by the conventional methods (tlc and partition chromatography).

The Merrifield method of solid-phase synthesis was found to be a convenient, fast, and reliable approach for the preparation of easily purified tripeptides starting with the benzhydrylamine resin described by Pietta.[7] The usual steps of coupling, deprotection, and neutralization[8] were accomplished employing BOC-proline, BOC-1-methylhistidine, BOC-3-methylhistidine, BOC-δ-Z-ornithine, BOC-ε-Z-lysine, BOC-methionine, BOC-O-benzyltyrosine, BOC-nitroarginine, and *N*-carbobenzoxypyroglutamic acid at the appropriate coupling steps. Cleavage from the resin was accomplished by HF. This treatment yielded, as an average after complete purification (generally 2 steps) based on the proline attached on the resin, 30% of the desired tripeptides: *i. e.*, **1a**; **1b**; pGlu-Orn-Pro-NH_2 (**7**); pGlu-Lys-Pro-NH_2 (**8**); pGlu-Met-Pro-NH_2 (**9**); pGlu-Tyr-Pro-NH_2 (**10**); and pGlu-Arg-Pro-NH_2 (**11**). Characterization of the final products was done by amino acid analysis, nmr, and mass spectrometry. Tlc in different systems (basic, neutral, and acidic) indicated that the compounds were homogeneous.

Biological Activity. The biological activities as measured by the assay described by Vale, *et al.*,[9] are reported in Table I. The [3-Me-His²]-TRF (**1b**) synthesized by either classical or solid-phase method appears to be approximately 10 times more active than TRF, whereas the [1-Me-His²]-TRF (**1a**) had only 0.04% of the activity of TRF. [Orn²]-, [Lys²]-, [Tyr²]-, [Arg²]-TRF (**7**, **8**, **10**, **11**) respectively, are somewhat active having less than 0.1% of the activity of TRF. Bowers, *et al.*,[3c] reported that pGlu-1-benzylhistidylprolinamide had only around 0.2% of TRF activity. Of interest is the fact that [Met²]-TRF (**9**) has 1% of TRF activity. It should be noted that a dipeptide, pGlu-3-Me-His-OMe, shows some definite activity whereas pGlu-1-Me-His-OMe and its analog pGlu-His-OMe do not show any activities at the level that were assayed (less than 1/40,000 and 1/200,000 of TRF biological activity, respectively).

Discussion

The most likely explanation for the enhanced activity of [3-Me-His²]-TRF and low activity of [1-Me-His²]-TRF was presented by Guillemin, *et al.*,[10] and Vale, *et al.*[2] The increased biological activity of [3-Me-His²]-TRF is perhaps due to a greater binding affinity for the TRF receptor. This enhanced binding affinity might be explained on the basis of a higher p*K* of the 1-N (p*K* = 6.56) and/or a steric effect of the 3-Me residue whereas the low activity of the [1-Me-His²]-TRF may be explained by a negative influence of the increased electron density on the 3-N (p*K* = 6.48)§ and/or a steric hindrance. Hofmann and Bowers[3a] have reported the synthesis of the [pyrazolyl-3-alanine²]-TRF analog, and found that it has 5% of the TRF activity (p*K* ≃ 2.1 for N≤ of pyrazolyl-3-alanine).§ They concluded that the characteristic acid-base behavior of the imidazole ring of histidine was not an essential feature for TSH release.

As suggested by results described in Table 1, neither the aromatic property of the imidazole ring nor its basic character can be exclusively responsible for the overall activity of the natural product; indeed, (1) substitution of histidine by tyrosine does decrease considerably the activity of the corresponding tripeptide, and (2) substitution of histidine by either ornithine, lysine, arginine, and tyrosine [p*K* values 8.65 (NH_3^+), 8.90 (NH_3^+), 13.2 (guan), 10.07 (OH), respectively, compared with 6.00–6.10 for N≤ of His]§ also decreases considerably the activity. [Met²]-TRF in this series of analogs shows 1% of the natural TRF activity.

At this point, we can conclude that whatever the mechanism involved by these tripeptides or those described by other groups to trigger secretion of TSH, only one of them, namely [3-Me-His²]-TRF has so far appeared to be more active than the natural product.

Experimental Section#

tert-BOC-amino acids** were bought from Fox chemicals and further purified when necessary. All amino acids were of the L configuration.†† Ascending tlc was conducted on silica gel supported either on glass plates (F-254, Merck AG) or plastic sheets (type 6060 with fluorescent indicator from Eastman). All the prep tlc plates (Al_2O_3 F^{254} type T from Merck AG) were first run in MeOH and then reactivated for 4 hr at 110°. Solvent systems for tlc: (1) MeOH–$CHCl_3$, 1:1 (v/v); (2) *n*-BuOH–AcOH–H_2O, 4:1:5 (v/v, upper phase); (3) *n*-BuOH–EtOAc–0.2 *N* NH_3, 1:1:2 (v/v, upper phase); (4) *n*-BuOH–EtOAc–AcOH–H_2O, 1:1:1:1 (v/v). Partition chromatographies were performed on the same column, 0.9 × 45 cm, packed

‡According to the IUPAC–IUB Commission on Biochemical Nomenclature[6a] pGlu-1-Me-His-Pro-NH_2 will have the semitrivial name [2-1-methylhistidine]-TRF and be abbreviated [1-Me-His²]-TRF. pGlu stands for pyroglutamic acid.[6b]

§These values obtained from Schneider and Schalf[11a] and Edsall[11b] are those of the free amino acids; variations have been observed by Schneider[11c] and others when the amino acids were part of a sequence.

#Melting points (Thomas-Hoover capillary mp apparatus) are uncorrected. Ir data were recorded on Beckman IR-18A spectrophotometer (KBr pellets). The nmr spectra were obtained with a Jeolco JNM-PS-100 spectrometer. Chemical shifts were recorded downfield from internal TMS when $CDCl_3$ or DMSO-d_6 were used, or from SDSS (sodium 2,2-dimethyl-2-silapentanesulfonate) when D_2O was used (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet). Mass spectra were obtd at 70 eV with a Varian CH-5; data were recorded with the Varian 620 I computer. High-resoln mass spectrometry was used to characterize the final products as such for [1- and 3-Me-His²]-TRF, permethylated[12] for [Orn²-, Lys²-, Met²-, Tyr²]-TRF and as the [N^{δ}-2 (4,6-dimethylpyrimidyl)-Orn²]-TRF for [Arg²]-TRF. Amino acid analyses were performed on peptide hydrolysates (6 *N* HCl at 110° in evacuated sealed tubes for 20 hr) using a Beckman/Spinco, Model 120, amino acid analyzer. Peak areas were detd by an Infotronics Model CRS-100A electronic integrator. A short column (5.2 cm: spherical resin type PA-35) was used with a citrate buffer (0.2N) pH 4.25 to analyze 1-Me-His, 1,3-Me_2-His, His, and 3-Me-His, (resp retention times 59, 65, 68, and 70 min). Loads of up to 4500 nmole were applied allowing us to detect less than 0.5% of any contaminant.

**BOC-3-Me-L-histidine, α-BOC-ε-Z-L-lysine, α-BOC-δ-Z-L-ornithine, BOC-*O*-benzyl-L-tyrosine. BOC-1-Me-L-histidine contained 0.7% of BOC-3-Me-L-histidine + an unknown contaminant. *tert*-BOC-NO_2-L-arginine contd an unidentified amino acid (*tert*-BOC-ornithine?) eliminated by repeated extn in boiling $CHCl_3$. pGlu was introduced as Z-pGlu; Z = carbobenzoxy.

††The final products were not analyzed for racemization.

Table I. *In Vivo* Biological Activity[a] of Synthetic TRF Analogs

No.	Compd	U/mg
1	pGlu-His-Pro-NH_2	50,000
1a	pGlu-1-Me-His-Pro-NH_2	20
1b	pGlu-3-Me-His-Pro-NH_2	400,000
5	pGlu-His-OMe	<0.25
5a	pGlu-1-Me-His-OMe	<1.25
5b	pGlu-3-Me-His-OMe	10
7	pGlu-Orn-Pro-NH_2	12.5
8	pGlu-Lys-Pro-NH_2	10
9	pGlu-Met-Pro-NH_2	500
10	pGlu-Tyr-Pro-NH_2	42.0
11	pGlu-Arg-Pro-NH_2	25.0

[a]The biological activities were measured in mice by the assay described by Vale, *et al.*[9]

with Sephadex G-25 F in 0.2 *N* AcOH. It was satd with the lower phase of either system described and then equilibrated with the upper phase. The sample was then applied as a soln in a maximum of 1 ml of upper phase: aliquots of 1 ml were collected. Uv, I_2, ninhydrin spray, and Pauly reagent (Stahl)[13] were successively used to examine tlc plates. A modification of the phenanthrenequinone test[14]‡‡ was applied for arginine. Only the positive tests will be mentioned. All the new peptides appeared homogeneous under these condns. Peptide yields, where indicated, are calcd on the basis of mmoles of peptide isolated after final purification relative to the total mmoles of starting BOC-amino acid, *viz.*, as resin amide.

1-Me-His-OMe · 2HCl (2a) or 3-Me-His-OMe · 2HCl (2b) were obtd by following the standard procedure described for histidine.[15] On a 10 mmoles (1.69 g of starting free amino acids) scale, yields were 2.25 g (89%) and 2.30 g (90%), respectively.

2a had mp 201° ($MeOH-Et_2O$); ir (KBr) 1740 (ester C=O); nmr (D_2O) δ 8.52 (s, 1, Im-2-H-1-Me-His), 7.27 (s, 1, Im-4-H-1-Me-His), 4.37 (t, 1, α-*H*-1-Me-His, $J_{\alpha,\beta}$ = 6.0 Hz, $J_{\alpha,\beta'}$ = 7.0 Hz), 3.72 (s, 3, Im-1-NCH_3), 3.68 (s, 3, OCH_3), 3.43 (dd, 1, β-CH_2-1-Me-His, $J_{\beta\beta'}$ = 15.0 Hz), 3.28 (dd, 1, β'-CH_2-1-Me-His); mass spectrum *m/e* (rel intensity) 183 (18), 149 (1), 124 (60), 97 (58), 96 (100), 95 (100), 88 (51), 81 (50), 68 (56), 54 (56), 42 (61). *Anal.* Calcd mass for $C_{18}H_{13}N_3O_2$: 183.101. Found: 183.101.

2b had mp 208–209°($MeOH-Et_2O$); ir (KBr) 1745 (ester C=O); nmr (D_2O) δ 8.48 (s, 1, Im-2-H-3-Me-His), 7.22 (s, 1, Im-4-H-3-Me-His), 4.36 (t, 1, α-H-3-Me-His, $J_{\alpha,\beta} = J_{\alpha,\beta'}$ = 7.0 Hz), 3.72 (s, 3, Im-3-NCH_3), 3.68 (s, 3, OCH_3), 3.30 (d, 2, ββ'-CH_2-3-Me-His); mass spectrum *m/e* (rel intensity) 183 (11), 149 (1), 124 (94), 97 (56), 96 (100), 95 (89), 83 (60), 81 (85), 68 (55), 56 (50), 54 (51), 42 (63). *Anal.* Calcd mass for $C_{18}H_{13}N_3O_2$: 183.101. Found: 183.101.

Z-pGlu-1-Me-His-OMe (3a) or Z-pGlu-3-Me-His-OMe (3b). (2a) or (2b) (2.04 g; 8 mmoles), neutralized with NaOMe in MeOH, was coupled to Z-pGlu in CH_3CN as described by Gillessen, *et al.*[4]

3a (2.05 g, 60%) crystd in $MeOH-Et_2O$: mp 140–141°; ir (KBr) 1745 (ester C=O), 1721 (Z C=O), 1650 (NHC=O); nmr ($CDCl_3$) δ 7.78 (d, 1, NH), 7.25–7.00 (m, 5, Z-arom H), 6.94 (s, 1, Im-2-H-1-Me-His). 6.34 (s. 1, Im-4-H-1-Me-His), 5.02 (s, 2, Z-CH_2), 4.68 (dt, 1, α-H-1-Me-His, J_α, NH = 8.0 Hz, $J_{\alpha,\beta} = J_{\alpha,\beta'}$ = 5.0 Hz), 4.50 (m, 1, α-H-pGlu), 3.55 (s, 3, NCH_3), 3.24 (s, 3, OCH_3), 3.08 (m, 2, ββ'-CH_2-1-Me-His), 2.80–1.80 (m, 4, ββ'- and γγ'-pGlu); mass spectrum *m/e* (rel intensity) 428 (<1), 369 (<1), 320 (7), 261 (7), 180 (8), 166 (58), 135 (34), 108 (82), 107 (72), 95 (60), 79 (97), 77 (75), 65 (49), 51 (58), 44 (59). *Anal.* Calcd mass for $C_{21}H_{24}N_4O_6$: 428.170. Found: 428.173.

3b was chromatd on silica gel (1.5 × 40 cm). Elution with $CHCl_3$ eliminated all the uv-positive impurities. 3b was recovered after changing to a system of $CHCl_3$–MeOH (1:1) but it resisted crystn (1.80 g, 53%); nmr ($CDCl_3$) δ 7.20–6.90 (m, 7, 5 arom H + NH + Im-2-H-3-Me-His), 6.40 (s, 1, Im-4-H-3-Me-His), 4.88 (s, 2, Z-CH_2), 4.52 (t, 1, α-H-3-Me-His), 4.12 (m, 1, α-H-pGlu), 3.48 (s, 3, NCH_3), 3.38 (s, 3, OCH_3), 2.85 (d, 2, ββ'-CH_2-3-Me-His, $J_{\alpha,\beta} = J_{\alpha,\beta'}$ = 5.0 Hz), 2.40–1.70 (m, 4, ββ'- and γγ'-pGlu); mass spectrum identical with that of 3a.

pGlu-1-Me-His-OMe (5a) or pGlu-3-Me-His-OMe (5b). Complete catalytic hydrogenolysis of the Z group on 10% Pd/C of 3a or 3b (946 mg, 2 mmoles) took place in MeOH (30 ml) under vigorous shaking and a continuing flow of H_2 within 10 min.

5a (570 mg, 97%) crystd in $MeOH-Et_2O$: mp 179–180°; ir 1750 (ester C=O), 1680 (pGlu C=O), 1670 (amide bond C=O); nmr ($CDCl_3$) δ 7.96 (d, 1, CONHCH, *J* = 8.0 Hz), 7.44 (s, 1, pGlu NH), 4.74 (q, 1, α-H-1-Me-His, $J_{\alpha,\beta} = J_{\alpha,\beta'}$ = 7.5 Hz), 3.98 (m, 1, α-H-pGlu), 3.60 (s, 3, NCH_3), 3.44 (s, 3, OCH_3), 3.00 (d, 2, ββ'-CH_2-1-Me-His), 2.50–1.85 (m, 4, ββ'- and γγ'-pGlu); mass spectrum *m/e* (rel intensity) 294 (43), 235 (37), 210 (11), 166 (100), 135 (55), 96 (80), 95 (97), 44 (58). *Anal.* Calcd mass for $C_{13}H_{18}N_4O_4$: 294.133. Found: 294.132.

5b was obtd as an oil (575 mg, 98%) which did not cryst; nmr ($CDCl_3$ after exchange with D_2O) δ 7.16 (s, 1, Im-2-H-3-Me-His), 6.45 (s, 1, Im-4-H-3-Me-His), 4.56 (t, 1, α-H-3-Me-His, $J_{\alpha,\beta} = J_{\alpha,\beta'}$ = 5.5 Hz), 4.02 (m, 1, α-H-pGlu), 3.52 (s, 3, NCH_3), 3.44 (s, 3, OCH_3), 2.90 (d, 2, ββ'-CH_2-3-Me-His), 2.50–1.80 (m, 4, ββ'- and γγ'-pGlu); mass spectrum *m/e* (rel intensity) 294 (31), 208 (55), 166 (50), 127 (63), 110 (75), 84 (63), 60 (58), 56 (64), 45 (100), 43 (59), *Anal.* Calcd mass for $C_{13}H_{18}N_4O_6$: 294.133. Found: 294.132.

pGlu-1-Me-His-$NHNH_2$ (6a) or pGlu-3-Me-His-$NHNH_2$ (6b). To 5a or 5b (590 mg; 2 mmoles) in MeOH (10 ml) was added at −10° a 2-fold excess of hydrazine hydrate. The soln was stored overnight at 5°.

6a crystd: dec over 242°; ir (KBr) 1696 (hydrazide C=O), 1650 (amides C=O); nmr (D_2O) δ 7.26 (s, 1, Im-2-H-1-Me-His), 6.48 (s, 1, Im-4-H-1-Me-His), 4.36 (q, 1, α-H-1-Me-His, $J_{\alpha,\beta}$ = 7.0 Hz, $J_{\alpha,\beta'}$ = 8.0 Hz), 4.08 (m, 1, α-H-pGlu), 3.40 (s, 3, NCH_3), 3.00 (dd, 1, β-CH_2-1-Me-His, $J_{\beta,\beta'}$ = 15.0 Hz), 2.86 (dd, 1, β'-CH_2-1-Me-His), 2.55–1.50 (m, 4, ββ'- and γγ'-pGlu); amino acid analysis Glu: 1.00, 1-Me-His: 0.85; mass spectrum *m/e* (rel intensity) 294 (20), 235 (47), 210 (28), 151 (55), 124 (57), 96 (76), 95 (100), 84 (62).

6b crystd: dec over 226°; ir (KBr) 1680 (hydrazide C=O), 1650 (amide C=O); nmr (D_2O) δ 7.22 (s, 1, Im-2-H-3-Me-His), 6.60 (s, 1, Im-4-H-3-Me-His), 4.32 (q, 1, α-H-3-Me-His, $J_{\alpha,\beta} = J_{\alpha,\beta'}$ = 6.0 Hz), 4.10 (m, 1, α-H-pGlu), 3.45 (s, 3, NCH_3), 2.92 (dd, 1, β-CH_2-3-Me-His, $J_{\beta,\beta'}$ = 14.0 Hz), 2.78 (dd, 1, β'-CH_2-3-Me-His), 2.50–1.60 (m, 4, ββ'- and γγ'-pGlu); amino acid analysis Glu: 1.00, 3-Me-His: 0.94; mass spectrum *m/e* (rel intensity) 294 (17), 263 (33), 235 (50), 210 (52), 151 (75), 150 (75), 124 (100), 96 (96), 95 (95), 84 (71), 82 (72), 43 (80), 41 (81).

pGlu-1-Me-His-Pro-NH_2 (1a) [1-Me-His²]-TRF or pGlu-3-Me-His-Pro-NH_2 (1b) [3-Me-His²]-TRF. 6a or 6b (590 mg; 2 mmoles) was converted to its azide and was treated with prolinamide as described by Gillessen, *et al.*[4] Yields of 50% or a little higher were obtd after 2 steps of purification. 1a or 1b was first applied on a prep tlc plate and run with a mixt of $MeOH-CHCl_3$ (1:1). The observed R_f values, obtd by treating a strip of the plate with I_2, were, respectively, 0.60–0.50 and 0.70–0.55. Recovery of the products was made by successive extns of the scratched and pulverized Al_2O_3 with $MeOH-CHCl_3$ (1:1). The almost pure tripeptides were further chromatogd in the partition system no. 2. The analogs were collected over 20 ml (tubes 88–108 and 84–104, resp), each tube was analyzed by tlc (I_2 test), and the pure fractions were pooled (tubes 90–105 for [1-Me-His²]-TRF and tubes 86–100 for [3-Me-His²]-TRF) yielding the pure analogs to be described as the acetate salts.

1a had nmr (D_2O) δ 7.68 (s, 1, Im-2-H-1-Me-His), 6.85 (s, 1, Im-4-H-1-Me-His), 5.33 (q, 1, α-*H*-1-Me-His, $J_{\alpha,\beta}$ = 7.0 Hz, $J_{\alpha,\beta'}$ = 8.0 Hz), 4.50–4.10 (m, 2, α-H-Pro and α-H-pGlu), 3.80–3.40 (m, 2, δδ'-CH_2-Pro), 3.63 (s, 3, NCH_3), 3.03 (m, 2, ββ'-CH_2-1-Me-His), 2.40–1.60 (m, 8, ββ'- + γγ'-CH_2-pGlu and ββ'- + γγ'-CH_2-Pro); amino acid analysis 1-Me-His: 1.00, NH_3: 1.34, Glu: 1.02, Pro: 1.08; mass spectrum *m/e* (rel intensity) 376 (7), 263 (13), 248 (65), 235 (54), 204 (50), 153 (43), 135 (52), 97 (65), 96 (100), 95 (93), 84 (95), 70 (85), 44 (46). *Anal.* Calcd mass for $C_{17}H_{24}N_6O_4$: 376.186. Found: 376.186.

1b had nmr (D_2O) δ 7.42 (s, 1, Im-2-H-3-Me-His), 6.80 (s, 1, Im-4-H-3-Me-His), 5.32 (t, 1, α-H-3-Me-His, $J_{\alpha,\beta} \cong J_{\alpha,\beta'}$ = 8.0 Hz), 4.30–4.00 (m, 2, α-H-Pro and α-H-pGlu), 3.80–3.10 (m, 2, δδ'-CH_2-Pro), 3.48 (s, 3, NCH_3), 2.88 (dd, 1, β-CH_2-3-Me-His, $J_{\beta,\beta'}$ = 14.0 Hz), 2.72 (dd, 1, β'-CH_2-3-Me-His), 2.40–1.50 (m, 8, ββ'- + γγ'-CH_2-pGlu and ββ'- + γγ'-CH_2-Pro); amino acid analysis 3-Me-His: 0.96, NH_3: 3.06, Glu: 1.00, Pro: 1.00; mass spectrum *m/e* (rel intensity) 376 (<1), 263 (3), 248 (25), 205 (6), 150 (30), 129 (37), 108 (53), 97 (70), 96 (100), 95 (75), 84 (78), 81 (55), 70 (81), 68 (62), 56 (52), 42 (67), 41 (71), 40 (80). *Anal.* Calcd mass for $C_{17}H_{24}N_6O_4$: 376.186. Found: 376.187.

Solid Phase. All the syntheses were done on a benzhydrylamine resin as described by Pietta and Marshall.[7] Different modifications of its prepn and a standard deblocking procedure (50% TFA–CH_2Cl_2 + 5% 2-ethanedithiol) as well as coupling procedure (DCI), and cleavage from the resin (HF) will be described elsewhere.

Purification. The crude products obtd after cleavage from the resin were estimated to be more than 75% pure on the basis of tlc and nmr spectra. Mass spectra showed the correct *m/e* except for

‡‡The chromatograms were sprayed with the phenanthrene quinone reagent instead of being dipped into it.

[Arg^2]-TRF for which derivatization (acetylacetone condn with the guanidino group) was necessary.[16]

[1-Me-His]-TRF (1a) and [3-Me-His]-TRF (1b) (around 30 mg of each) were purified following the redundant procedure already described. Yields were acceptable even when only 2 mequiv of *tert*-BOC derivatives were added per mequiv of free proline resin amide. Usually a 10-fold excess of the reagent allowed us to shorten the reaction time considerably.

[Ornithine^2]-TRF (7) and [lysine^2]-TRF (8) (around 50 mg of each) were purified on a partition column (system 2) as aforementioned. The products were collected over 30 ml and 22 ml (fractions 90–120 and fractions 86–108, respectively. Fractions 96–107 yielded pure [Orn^2]-TRF as its acetate salt and fractions 88–97 yielded pure [Lys^2]-TRF as its acetate salts (I_2 and ninhydrin tests).

7 was obtained in 32% yield; nmr (D_2O) δ 4.70 (m, 1, α-H-Orn). 4.50–4.20 (m, 2, α-H-pGlu and α-H-Pro), 3.90–3.50 (m, 2, δδ′-CH_2-Pro), 3.00 (m, 2, ββ′-CH_2-Orn), 2.70–1.60 (m, 12, ββ′- + γγ′-pGlu and ββ′- + γγ′-Pro and ββ′- + γγ′-Orn), 1.92 (s, 3, acetate salt); amino acid analysis Orn: 0.92, NH_3: 0.86, Glu: 1.00, Pro: 0.93; mass spectrum *m/e* (rel intensity) 339 (<1), 322 (<1), 275 (1), 225 (6), 192 (11), 182 (120), 141 (30), 113 (31), 99 (78), 84 (61), 70 (100), 60 (59), 55 (50), 49 (68), 43 (83), 41 (70). *Anal.* Calcd mass for $C_{21}H_{37}N_5O_4$: 423.285. Found: 423.287.

8 was obtained in 33% yield; nmr (D_2O) δ 4.58 (m, 1, α-H-Lys), 4.40–4.20 (m, 2, α-H-pGlu and α-H-Pro), 4.00–3.40 (m, 2, δδ′-CH_2-Pro), 3.10–2.80 (εε′-CH_2-Lys), 2.60–1.20 (m, 14, ββ′- + γγ′-pGlu and ββ′- + γγ′-Pro and ββ′- + γγ′- + δδ′-Lys), 1.90 (s, 3, acetate salt); amino acid analysis Lys: 0.95, NH_3: 1.04, Glu: 1.00, Pro: 1.00; mass spectrum *m/e* (rel intensity) 353 (<1), 310 (<1), 282 (2), 240 (15), 225 (9), 208 (5), 167 (9), 155 (19), 129 (12), 115 (19), 84 (85), 70 (86), 61 (59), 60 (99), 56 (55), 55 (100). *Anal.* Calcd mass for $C_{22}H_{39}N_5O_4$: 437.300. Found: 437.303.

[Methionine^2]-TRF (9) and [tyrosine^2]-TRF (10) (around 50 mg of each) were purified on a partition column (system 3). The products were collected over 20-ml fractions, 80–100 and fractions 85–105. Fractions 84–96 yielded pure [methionine^2]-TRF (I_2 test) and 91–103 yielded pure [tyrosine^2]-TRF (I_2, uv and Pauly tests).

9 was obtained in 22% yield; nmr (D_2O) δ 4.60 (q, 1, α-H-Met, $J_{\alpha,\beta} + J_{\alpha,\beta'}$ = 15.0 Hz), 4.25–4.00 (m, 2, α-H-pGlu + α-H-Pro), 3.80–3.40 (m, 2, δδ′-CH_2-Pro), 2.70–1.70 (m, 12, ββ′- + γγ′-Met and ββ′- + γγ′-pGlu and ββ′- + γγ′-Pro), 2.05 (s, 3, SCH_3); amino acid analysis NH_3: 1.09, Glu: 1.00, Pro: 0.93, Met: 0.94; mass spectrum *m/e* (rel intensity): 356 (<1), 339 (<1), 312 (<1), 282 (47), 254 (38), 229 (43), 228 (54), 199 (4), 181 (35), 168 (51), 165 (57), 155 (66), 154 (99), 139 (51), 115 (50), 85 (60), 84 (100), 70 (100), 42 (100). *Anal.* Calcd mass for $C_{19}H_{32}N_4O_4S$: 412.214. Found: 412.217.

10 was obtained in 24% yield; nmr (D_2O) δ 7.10 (m, 2, arom), 6.82 (d, 2, arom $J_{2,3}$ = 9.0 Hz), 4.85 (m, 1, α-H-Tyr), 4.40–4.10 (m, 2, α-H-pGlu + α-H-Pro), 3.95–3.25 (m, 2, δδ′-CH_2-Pro), 3.10–2.70 (m, 2, ββ′-CH_2-Tyr), 2.50–1.50 (m, 8, ββ′- + γγ′-pGlu and ββ′- + γγ′-Pro); amino acid analysis NH_3: 1.07, Glu: 1.00, Pro: 0.97, Tyr: 0.92; mass spectrum *m/e* (rel intensity) 388 (<1), 350 (4), 282 (8), 261 (28), 260 (61), 216 (40), 197 (38), 168 (55), 155 (71), 154 (100). 125 (55), 108 (65), 107 (<100), 84 (<100), 70 (<100). *Anal.* Calcd mass for $C_{24}H_{34}N_4O_5$: 458.253. Found: 458.254.

[Arginine^2]-TRF (11) was applied on a prep tlc plate and run with $CHCl_3$–MeOH–AcOH (90:8:2). The product appeared below a strong uv-positive line (R_f 0.2) and appeared yellow when sprayed with Pauly's reagent. It was carefully collected and chromatogd a second time on a prep tlc plate ($CHCl_3$–MeOH–AcOH; 85:10:5). A cut in the middle of the Pauly- and I_2-positive region (R_f 0.3) yielded after extn the pure analog as its acetate salt; yield 28%; nmr (D_2O) δ 4.60 (q, 1, α-H-Arg, $J_{\alpha,\beta} + J_{\alpha,\beta'}$ = 14.0 Hz), 4.45–4.20 (m, 2, α-H-pGlu and α-H-Pro), 3.90–3.45 (m, 2, δδ′-CH_2-Pro), 3.35–3.10 (m, 2, δδ′-CH_2-Arg), 2.70–1.60 (m, 8, ββ′- + γγ′-pGlu and ββ′- + γγ′-Pro and ββ′- + γγ′-Arg), 2.00 (s, 3, acetate); amino acid analysis NH_3: 1.01, Arg: 1.01, Glu: 1.00, Pro: 1.00; mass spectrum of [*N*-2-(4,6-dimethylpyrimidyl)Orn^2]-TRF: *m/e* (rel intensity) 445 (3), 361 (2), 332 (8), 304 (5), 248 (40), 176 (51), 150 (53), 136 (52), 99 (54), 96 (82), 95 (100), 84 (79), 70 (73), 68 (51), 44 (58), 43 (56), 42 (50), 41 (66). *Anal.* Calcd mass for $C_{21}H_{31}N_7O_4$: 445.244. Found: 445.247.

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