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Synthesis and Biological Activity of 8-L-Ala-Motilin and 8-D-Ala-Motilin[1]

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Two analogs of porcine motilin altered at position 8, [L-Ala[8]]-motilin and [D-Ala[8]]-motilin, were prepared by the conventional solution method *via* the corresponding sulfoxides (Met[13]) and evaluated *in vitro* for the ability to contract the duodenal muscle of rabbit. The D-Ala analog exhibited a higher potency than synthetic motilin, whereas substitution of glycine at position 8 with L-alanine decreased the biological activity to approximately 30% relative to the synthetic motilin.

Keywords——motilin; motilin analog; D-Ala[8]-motilin; docosapeptide; methanesulfonic acid; Type II β-bend conformation; rabbit duodenal muscle

Since the elucidation of the complete amino acid sequence of porcine motilin (H–Phe–Val–Pro–Ile–Phe–Thr–Tyr–Gly–Glu–Leu–Gln–Arg–Met–Gln–Glu–Lys–Glu–Arg–Asn–Lys–Gly–Gln–OH),[3] several syntheses of this gastric motor activity-stimulating polypeptide by solution method[4,5] and solid phase procedure[6] have been reported. We have also reported an alternate synthesis of this hormone using methanesulfonic acid as a deprotecting reagent at the final step of the synthesis.[7] With regard to the syntheses of motilin analogs, only four, [Leu[13], Glu[14]]- and [norleucine[13], Glu[14]]- motilin by Wünsch, *et al.*[8] and [D-Phe[1]]- and [Met(O)[13]]-motilin by Fujino, *et al.*,[7] have been reported to date.

In order to determine the structure-activity relationships in the motilin molecule and search for a potent agonist, we attempted to prepare [D-Ala[8]]-motilin in which D-alanine was incorporated in the position 8 (glycine), because synthetic studies on analogs of luteinizing

1) Amino acids, peptides and their derivatives in this paper are of the L-configuration unless otherwise mentioned. The following abbreviations are used: Z=benzyloxycarbonyl, BOC=*tert*-butoxycarbonyl, MBS=*p*-methoxybenzenesulfonyl, OBu^t=*tert*-butyl ester, OBzl=benzyl ester, HONB=N-hydroxy-5-norbornene-2,3-dicarboximide, DCC=N,N′-dicyclohexylcarbodiimide, DCU=N,N′-dicyclohexylurea, TFA=trifluoroacetic acid, TEA=triethylamine, DMF=N,N-dimethylformamide, THF=tetrahydrofuran.

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hormone-releasing hormone (LH-RH)[9] and enkephalin[10] have shown that replacement of glycine adjacent to tyrosine by D-alanine yields a super active agonist.

In this paper we report the synthesis and biological activity of two analogs of motilin, [D-Ala^8]-motilin and [L-Ala^8]-motilin.

The analogs were synthesized in a manner similar to that described for our synthesis of porcine motilin.[7] They were prepared *via* the corresponding sulfoxide (Met^{13}) to prevent side reactions.[11] Three key intermediates, BOC-Lys(Z)-Glu(OBzl)-Arg(MBS)-Asn-Lys(Z)-Gly-Gln-OBzl (I),[7] BOC-X-Glu(OBzl)-Leu-Gln-Arg(MBS)-Met(O)-Gln-Glu(OBzl)-OH [(II-a): X=L-Ala, (II-b): X=D-Ala] and BOC-Phe-Val-Pro-Ile-Phe-Thr-Tyr-OH (III), were prepared in a stepwise chain elongation manner using the HONB active ester of N-protected amino acids.[12] As shown in Fig. 1, the C-terminal fragment I was treated with

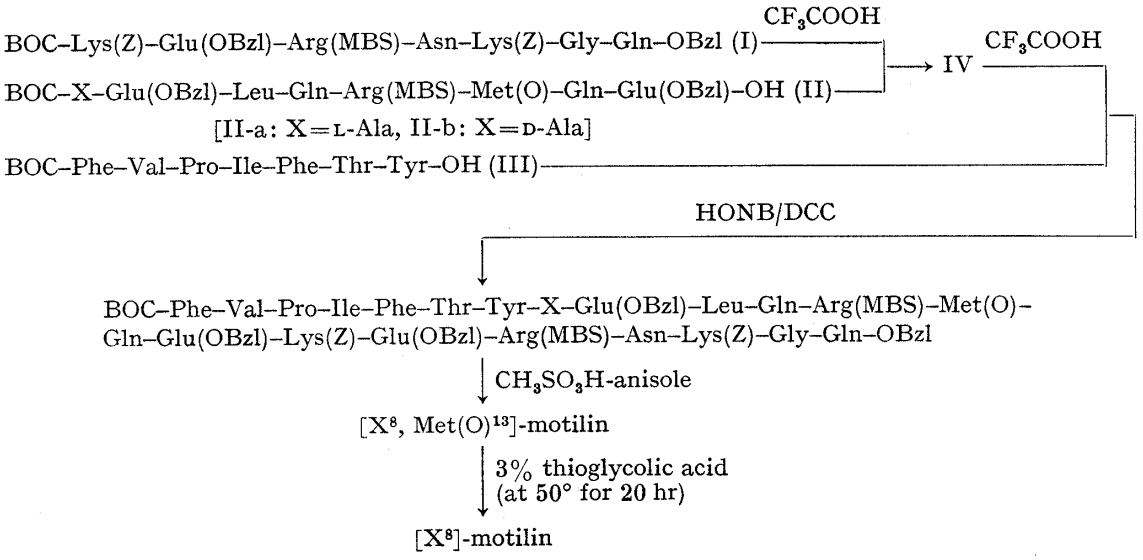

Fig. 1. Synthetic Route to [L-Ala^8]- or [D-Ala^8]-Motilin
X=L-Ala or D-Ala

TFA to remove the N$^{\alpha}$-BOC protecting group and the resulting free base of I was coupled to fragment II by the HONB-DCC method to avoid undesirable racemization during the coupling reaction.[13] The protected pentadecapeptide [(IVa): X=L-Ala; (IVb): X=D-Ala] thus obtained was treated with TFA, and the resulting free base was condensed with the N-terminal fragment III by DCC in the presence of HONB. The crude protected docosapeptide was treated with methanesulfonic acid-anisole[14] to remove all the protecting groups, followed by conversion to the corresponding acetate with Amberlite IRA-410 (acetate form). The acetate was purified by column chromatography on Sephadex LH-20 and subsequently on carboxymethylcellulose in a similar manner to that described for the purification of synthetic

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motilin.[7] The purified docosapeptide sulfoxide was exposed to 3% aqueous thioglycolic acid for 20 hr at 50°. The reduced peptide was then purified on a column of Sephadex LH-20 (30% acetic acid). The purified analogs were found to be homogeneous when checked by thin layer chromatography and paper electrophoresis, and the expected ratios were obtained from amino acid analyses of the acid hydrolysate.

Biological Evaluation and Discussion

Biological activities of [L-Ala[8]]-motilin and [D-Ala[8]]-motilin were compared with that of our synthetic motilin for their ability to contract rabbit duodenal muscle according to the method of Segawa, *et al.*[15] The results are presented in Table I. As expected, [D-Ala[8]]-motilin exhibited a somewhat high potency when compared with the parent hormone, whereas the L-alanine analog had a considerably lower activity than motilin. Though the D-alanine analog exhibited only a slightly higher potency *in vitro* than motilin, it might exhibit a more intense and prolonged action when administered in an *in vivo* system, because of its increased resistance to degradation by biological fluids.

Table I. Relative Contractile Activity of Synthetic Motilin Analogs on Rabbit Duodenal Muscle

Compound	Relative activity
Motilin	1
L-Ala[8]-Motilin	0.297 (0.277—0.324)
D-Ala[8]-Motilin	1.147 (0.788—1.303)

Data were assessed statistically by 4-point assay procedure.
Figures in parentheses are 95% confidence intervals.

Our results suggest that in receptor-hormone interaction the tertiary structure of D-alanine analog is more stable than that of the L-alanine analog or the parent molecule, and may be interpreted in terms of Type II β-bend conformation in the sequence of –Tyr[7]–Gly[8]–Glu[9]– as in the case of LH–RH and enkephalin.[9,10]

From our results and literature,[9,10] we concluded that substitution of glycine residue by D-alanine might give an agonist with potent activity when a biologically active peptide has a particular sequence of –Tyr–Gly– in the molecule.

Experimental

All melting points were taken in open capillaries and are uncorrected. Rotations were determined with a Perkin-Elmer Model 141 polarimeter. Amino acid analyses were performed on a Hitachi KLA-3B amino acid analyzer. Acid hydrolyses were carried out according to the method of Matsubara and Sasaki.[16] Evaporations were carried out in a rotary evaporator under reduced pressure at a temperature of 40—45°. Catalytic hydrogenations were performed at room temperature with palladium black as catalyst. The purity of the products was tested by thin-layer chromatography (TLC) using Merck precoated silica gel plate $60F_{254}$. Solvent systems used were: $CHCl_3$–MeOH–AcOH (9: 1: 0.5, Rf^1), AcOEt–pyridine–AcOH–H_2O (30: 10: 3: 5, Rf^2), *n*-BuOH–pyridine–AcOH–H_2O (30: 20: 6: 24, Rf^3), *n*-BuOH–AcOEt–AcOH–H_2O (1: 1: 1: 1, Rf^4), *n*-BuOH–AcOH–H_2O (4: 1: 5, upper phase, Rf^5), $CHCl_3$–MeOH–AcOH (8: 2: 0.5, Rf^6).

BOC-Ala-Glu(OBzl)-Leu-Gln-Arg(MBS)-Met(O)-Gln-Glu(OBzl)-OH (IIa)——BOC–Glu(OBzl)–Leu–Gln–Arg(MBS)–Met(O)–Gln–Glu(OBzl)–OH[7] (0.60 g) was treated with TFA (5 ml) for 20 min at 20°. After evaporation the residue was triturated with dry ether. The precipitate was collected and dried over NaOH pellets *in vacuo*. A part of the powder (0.30 g, 0.21 mmol) was dissolved in DMF (10 ml) together with TEA (0.06 ml). To this was added BOC–Ala–ONB (105 mg, 0.3 mmol), which was prepared by the DCC method. The mixture was stirred for 12 hr at room temperature and to this was then added AcOH (0.1 ml). After evaporation, the residue was triturated with ether and then purified by reprecipitation from DMF-ether: 0.27 g (87%), mp 193—195° (dec.), $[\alpha]_D^{24}$ −14.0° (c=0.5 in DMF), Rf^2=0.24, Rf^5=0.40, Rf^6=0.15.

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Anal. Calcd. for $C_{66}H_{95}N_{13}O_{21}S_2 \cdot 2H_2O$: C, 52.60; H, 6.62; N, 12.08; S, 4.25. Found: C, 52.58; H, 6.51; N, 12.01; S, 4.13.

BOC-D-Ala-Glu(OBzl)-Leu-Gln-Arg(MBS)-Met(O)-Gln-Glu(OBzl)-OH (IIb)——The deblocked heptapeptide (0.30 g, 0.21 mol) was coupled with BOC-D-Ala-ONB (105 mg, 0.3 mmol) as described for the preparation of compound IIa to give the desired compound: 268 mg (86%), mp 192—195° (dec.), $[\alpha]_D^{24}$ −9.6° (c=0.5 in DMF), Rf^2=0.24, Rf^5=0.40, Rf^6=0.15. *Anal.* Calcd. for $C_{66}H_{95}N_{13}O_{21}S_2 \cdot 2H_2O$: C, 52.60; H, 6.62; N, 12.08; S, 4.25. Found: C, 52.71; H, 6.48; N, 11.94; S, 4.08.

BOC-Phe-Thr-Tyr-OBzl (III-1)——To a solution of H-Tyr-OBzl *p*-toluenesulfonate (4.0 g, 9 mmol) and TEA (1.26 ml) in THF (100 ml) were added BOC-Thr-OH (1.97 g, 9 mmol) and DCC (2.04 g) at 0°. The solution was stirred for 12 hr at 20° and then filtered to remove the formed DCU. The filtrate was evaporated and the residue was dissolved in AcOEt (200 ml). The AcOEt solution was washed with 0.1 N HCl and 5% $NaHCO_3$, dried over anhydr. Na_2SO_4. After evaporation the residue was treated with TFA (20 ml) for 20 min at 20°. The mixture was evaporated and the residue was washed with dry ether. The residue was dried over NaOH pellets *in vacuo* and then dissolved in THF (50 ml) together with TEA (1.2 ml). To this was added BOC-Phe-ONB (3.40 g, 8 mmol) at 0°. After stirring for 12 hr and the usual work-up, the material was crystallized from ether-pet. ether: 4.8 g, (86%), mp 141—143°, $[\alpha]_D^{24}$ −5.4° (c=0.5 in DMF), Rf^1=0.46. *Anal.* Calcd. for $C_{34}H_{41}N_3O_8 \cdot H_2O$: C, 64.03; H, 6.79; N, 6.59. Found: C, 64.19; H, 6.67; N, 6.67.

BOC-Ile-Phe-Thr-Tyr-OBzl (III-2)——Compound III-1 (3.1 g, 5.0 mmol) was treated with TFA (15 ml) for 20 min at 20°. The usual work-up provided a precipitation, which was dissolved in THF (30 ml) and to this was added TEA (0.7 ml) at 0°. To the solution was added BOC-Ile-ONB (2.04 g, 5.2 mmol) and the mixture was stirred for 12 hr at room temperature. The material isolated by the usual manner was purified by crystallization from AcOEt: 2.20 g (60%), mp 186—189°, $[\alpha]_D^{23}$ −7.4° (c=0.5 in DMF), Rf^1=0.68. *Anal.* Calcd. for $C_{40}H_{52}N_4O_9 \cdot 1/2H_2O$: C, 64.76; H, 7.20; N, 7.55. Found: C, 64.91; H, 7.30; N, 7.53.

BOC-Pro-Ile-Phe-Thr-Tyr-OBzl (III-3)——Compound III-2 (2.0 g, 2.73 mmol) was treated with TFA (10 ml) and the TFA salt isolated as a powder was dissolved in THF (20 ml) containing TEA (0.4 ml). To this was added BOC-Pro-ONB (1.02 g). The mixture was stirred for 12 hr at room temperature and then evaporated. The usual work-up gave a precipitate, which was purified by reprecipitation from MeOH-ether: 1.95 g (86%), mp 138—139°, $[\alpha]_D^{24}$ −27.2° (c=0.5 in DMF), Rf^1=0.73. *Anal.* Calcd. for $C_{45}H_{59}N_5O_{10}$: C, 65.11; H, 7.16; N, 8.43. Found: C, 64.71; H, 7.30; N, 8.25.

BOC-Val-Pro-Ile-Phe-Thr-Tyr-OBzl (III-4)——Compound III-3 (1.66 g, 2 mmol) was treated with TFA (10 ml) and the resulting TFA salt was acylated with BOC-Val-OH (434 mg) *via* the corresponding HONB ester in DMF (20 ml) for 48 hr. After the usual work-up, the substance was purified by reprecipitation from AcOEt-ether: 1.60 g (86%), mp 143—145°, $[\alpha]_D^{24}$ −35.8° (c=0.5 in DMF), Rf^1=0.62. *Anal.* Calcd. for $C_{50}H_{68}N_6O_{11} \cdot H_2O$: C, 63.40; H, 7.44; N, 8.87. Found: C, 63.80; H, 7.49; N, 9.11.

BOC-Phe-Val-Pro-Ile-Phe-Thr-Tyr-OBzl (III-5)——Compound III-4 (1.10 g, 1.2 mmol) was treated with TFA (5 ml), and the resulting product was coupled with BOC-Phe-ONB (532 mg) in DMF (10 ml) containing TEA (0.17 ml). After the usual work-up the material was purified by reprecipitation from *n*-BuOH-ether: 1.05 g (81%). mp 154—156°, $[\alpha]_D^{24}$ −38.0° (c=0.5 in DMF), Rf^1=0.66. *Anal.* Calcd. for $C_{59}H_{77}N_7O_{12}$: C, 64.75; H, 7.27; N, 8.96. Found: C, 64.98; H, 7.18; N, 9.00.

BOC-Phe-Val-Pro-Ile-Phe-Thr-Tyr-OH (III)——Compound III-5 (1.0 g, 0.92 mmol) was hydrogenated in MeOH (50 ml) to remove the benzyl ester. The reaction mixture was filtered to remove the catalyst and the filtrate was evaporated to dryness. The residue was then triturated with ether to give a fine powder: 0.90 g (99%), mp 175—177°, $[\alpha]_D^{24}$ −28.4° (c=0.5 in DMF), Rf^1=0.21. *Anal.* Calcd. for $C_{52}H_{71}O_{12}N_7 \cdot H_2O$: C, 62.19; H, 7.32; N, 9.76. Found: C, 62.10; H, 7.42; N, 9.93.

BOC-Ala-Glu(OBzl)-Leu-Gln-Arg(MBS)-Met(O)-Gln-Glu(OBzl)-Lys(Z)-Glu(OBzl)-Arg(MBS)-Asn-Lys(Z)-Gly-Gln-OBzl (IVa)——BOC-Lys(Z)-Glu(OBzl)-Arg(MBS)-Asn-Lys(Z)-Gly-Gln-OBzl (I)[7] (710 mg, 0.45 mmol) was treated with TFA (4 ml) for 15 min at 20°. The usual work-up provided a precipitate, which was dissolved in DMF (2 ml) containing TEA (0.12 ml). To the mixture was added dry ether, and the resulting precipitate was collected by filtration and dried *in vacuo*. A part of the powder (260 mg) was dissolved in DMF (5 ml) together with compound IIa (260 mg, 0.175 mmol) and HONB (65 mg). To this was added DCC (55 mg) at −5°, and the mixture was stirred for 3 hr at −5°. After stirring at 20° for 12 hr, the reaction mixture was filtered and the filtrate was evaporated to dryness. The residue was triturated with ether and then purified by reprecipitation from DMF-ether: 465 mg (90%), mp 220—221° (dec.), $[\alpha]_D^{24}$ −4.2° (c=0.5 in DMF), Rf^2=0.65, Rf^5=0.55, Rf^6=0.27. *Anal.* Calcd. for $C_{137}H_{185}N_{27}O_{39}S_3 \cdot 2H_2O$: C, 55.46; H, 6.42; N, 12.75; S, 3.24. Found: C, 55.30; H, 6.36; N, 12.40; S, 3.18.

BOC-D-Ala-Glu(OBzl)-Leu-Gln-Arg(MBS)-Met(O)-Gln-Glu(OBzl)-Lys(Z)-Glu(OBzl)-Arg(MBS)-Asn-Lys-(Z)-Gly-Gln-OBzl (IVb)——The deblocked heptapeptide (250 mg, 0.17 mmol) derived from compound I was coupled with compound IIb (250 mg, 0.17 mmol) by the HONB-DCC method as described for the preparation of compound IVa to give the crude product, which was purified by reprecipitation from DMF-ether: 420 mg (84%), mp 220—223° (dec.), $[\alpha]_D^{24}$ −3.0° (c=0.5 in DMF), Rf^2=0.65, Rf^5=0.55, Rf^6=0.27. *Anal.* Calcd. for $C_{137}H_{185}N_{27}O_{39}S_3 \cdot 2H_2O$: C, 55.46; H, 6.42; N, 12.75; S, 3.24. Found: C, 55.11; H, 6.39; N, 12.61; S, 3.14.

[Ala^8, Met(O)^{13}]-Motilin——Compound IVa (381 mg, 0.13 mmol) was treated with TFA (2 ml) for 20 min at room temperature. The usual work-up provided a powder, which was dissolved in DMF (2 ml) together with TEA (0.1 ml). To this mixture was added dry ether to give a powder which was collected by filtration and dried. The powder was dissolved in DMF (3 ml) together with compound III (140 mg, 0.14 mmol) and HONB (93 mg), and to this was added DCC (82 mg) at −5°. After stirring at −5° for 3 hr and at room temperature for 12 hr, the mixture was filtered and evaporated. The residue was triturated with ether to give a powder: 410 mg. The powder (350 mg) was treated with methanesulfonic acid (5 ml) in the presence of anisole (0.7 ml) at room temperature for 60 min. To the reaction mixture was added dry ether (50 ml) at 0°, and the resulting precipitate was washed with dry ether. The precipitate was dissolved in H_2O (50 ml) and the solution was passed through a column (1.5×6 cm) of Amberlite IRA-410 (AcO^-). The eluate and washings were combined and lyophilized: 300 mg. The powder was dissolved in 30% AcOH (2 ml) and the solution was applied to a column (3.4×60 cm) of Sephadex LH-20, which was eluted with 30% AcOH. The fractions (156—186 ml) were collected and lyophilized: 150 mg. The powder was dissolved in H_2O and the solution was applied to a column (1.5×20 cm) of CM-cellulose. The material was eluted with pH 6.8 ammonium acetate buffer, gradient elution: 0.005 M/0.20 M=300 ml/300 ml. The fractions (105—133 ml) containing the pure product (checked by TLC) were combined and lyophilized: 82 mg (26%), $[\alpha]_D^{20}$ −52.5° (c=0.39 in 5% AcOH), Rf^3 (cellulose)=0.52, Rf^4 (cellulose)=0.59, Rf^5=0.14. Amino acid ratios in acid hydrolysate: Lys 2.02, Arg 2.02, Asp 1.07, Thr 0.73, Glu 6.66, Pro 0.98, Gly 1.02, Ala 0.87. Val 0.79, Met 0.87, Ile 0.79, Leu 0.99, Tyr 0.85, Phe 1.47 (average recovery, 78%).

[Ala^8]-Motilin——[Ala^8, Met(O)^{13}]-Motilin (55 mg) was dissolved in 3% aqueous thioglycolic acid (4 ml) and the solution was kept to stand at 50° for 20 hr. The solution was applied to a column (3.4×60 cm) of Sephadex LH-20, which was eluted with 30% AcOH. The fractions (165—190 ml) were collected and lyophilized: 42 mg (76%), $[\alpha]_D^{25}$ −49.6° (c=0.45 in 5% AcOH), Rf^3 (cellulose)=0.57, Rf^4(cellulose)=0.61, Rf^5=0.16. Amino acid ratios in acid hydrolysate: Lys 1.92, Arg 1.80, Asp 1.23, Thr 0.91, Glu 5.96, Pro 0.87, Gly 1.06, Ala 1.00, Val 1.05, Met 0.91, Ile 0.96, Leu 1.02, Tyr 0.89, Phe 1.95 (average recovery, 78%).

[D-Ala^8, Met(O)^{13}]-Motilin——The deblocked pentadecapeptide (375 mg, 0.13 mmol) derived from compound IVb was coupled with compound III (154 mg, 0.16 mmol) by the HONB–DCC method as described for the preparation of [Ala^8, Met(O)^{13}]-motilin. The crude protected docosapeptide (270 mg) was treated with methanesulfonic acid (3 ml) in the presence of anisole (0.3 ml) at room temperature for 60 min. The product was purified on a Sephadex LH-20 column and a CM-cellulose column in a manner similar to that described for [Ala^8, Met(O)^{13}]-motilin to give the desired product: 60 mg (20%), $[\alpha]_D^{20}$ −52.0° (c=0.25 in 5% AcOH), Rf^3 (cellulose)=0.52, Rf^4 (cellulose)=0.59, Rf^5 =0.14. Amino acid ratios in acid hydrolysate: Lys 2.11, Arg 2.16, Asp 1.08, Thr 0.91, Glu 6.04, Pro 1.03, Gly 1.13, Ala 0.99, Val 0.76, Met 0.85, Ile 0.84, Leu 1.10, Tyr 0.98, Phe 1.66 (average recovery, 73%).

[D-Ala^8]-Motilin——[D-Ala^8, Met(O)^{13}]-Motilin (33 mg) was treated with thioglycolic acid and the product was purified on a Sephadex LH-20 column as described for [Ala^8]-motilin to give the pure product: 20 mg (61%), $[\alpha]_D^{25}$ −48.1° (c=0.33 in 5% AcOH), Rf^3 (cellulose)=0.58, Rf^4 (cellulose)=0.61, Rf^5=0.16. Amino acid ratios in acid hydrolysate: Lys 2.10, Arg 2.12, Asp 1.07, Thr 0.89, Glu 5.91, Pro 0.95, Gly 1.00, Ala 1.04, Val 0.78, Met 0.89, Ile 0.84, Leu 0.89, Tyr 0.84, Phe 1.70 (average recovery, 75%).

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