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# Synthetic Studies on Enkephalin Analogs. I.[1] Potent Analgesic Activity of H-Tyr-D-Ala-Gly-Phe-NHNH-CO-R (R=Lower Alkyl)

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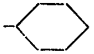
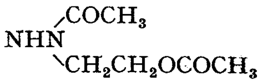
Thirty tetrapeptide hydrazide analogs of enkephalin, H–Tyr–D-Ala–Gly–Phe–NHN-($R_1$)–$R_2$ ($R_1$, $R_2$=H, alkyl or acyl), were synthesized. The analgesic activities of these peptides were tested in mice after intravenous or subcutaneous injection, and compared with that of morphine. Among the analogs synthesized, the tetrapeptide acyl-hydrazide analog, H–Tyr–D-Ala–Gly–Phe–NHNH–CO–R (R=lower alkyl), was the most active and showed analgesic activity ten times more potent than that of the tetrapeptide amide analog, H–Tyr–D-Ala–Gly–Phe–$NH_2$ and half as potent as that of morphine. On the basis of these results, structure-activity relations in the hydrazide part of enkephalin analogs of this new type are discussed.

**Keywords**——enkephalin; analgesia; morphine; tetrapeptide acyl-hydrazide; structure-activity relations

Since the isolation of a pentapeptide, enkephalin, with opiate-like activity from porcine brain by Hughes *et al.*,[2] several potent analogs of enkephalin such as [D-Met$^2$, Pro$^5$]-enkephalinamide,[3] [D-Ala$^2$, MePhe$^4$, Met(O)-ol$^5$]-enkephalin[4] and [D-Thr$^2$, Thz$^5$]-enkephalinamide[5] have been reported. Recently, McGregor *et al.*[6] reported that C-terminal amidation of the tetrapeptide H–Tyr–D-Ala–Gly–Phe–OH resulted in a remarkable increase in analgesic potency. Independently, we also observed analgesic activity of the tetrapeptide amide H–Tyr–D-Ala–Gly–Phe–$NH_2$ following subcutaneous administration. The enhancement of activity of the tetrapeptide by C-terminal amidation might be due to greater resistance of the C-terminal part to enzymatic degradation. This led us to attempt an alternative protection of the C-terminal carboxyl group of the tetrapeptide and to synthesize tetrapeptide hydrazide analogs of enkephalin in the hope of obtaining a more potent and useful analgesic. Some of these analogs were reported in a brief communication to have profound morphinomimetic activity.[7]

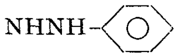
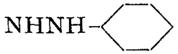
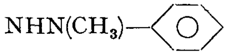
In the present series of three papers, we wish to describe our synthetic studies on the tetrapeptide hydrazide analogs of enkephalin in detail. This paper deals with the synthesis and biological evaluation of thirty analogs of enkephalin having D-Ala at position 2 and various kinds of hydrazides at position 5 (H–Tyr–D-Ala–Gly–Phe–X), carried out in order to obtain detailed information regarding structure-activity relations in the C-terminal part of the peptide. The synthesized analogs were I (X=$NHNH_2$), II (X=NHNH–$CH_3$), III (X=NHNH–$C_6H_5$), IV (X=NHNH–$C_6H_{11}$), V (X=NHN($CH_3$)–$C_6H_5$), VI (X=NHN($CH_3$)$_2$), VII (X=NHNH–CO–$CH_3$), VIII (X=NHNH–CO–$CH_2CH_3$), IX (X=NHNH–CO–($CH_2$)$_2$–$CH_3$), X (X=NHNH–CO–($CH_2$)$_3$–$CH_3$), XI (X=NHNH–CO–($CH_2$)$_4$–$CH_3$), XII (X=NHNH–CO–($CH_2$)$_5$–$CH_3$), XIII (X=NHNH–CO–CH($CH_3$)$_2$), XIV (X=NHNH–CO–$CH_2$CH($CH_3$)$_2$), XV (X=NHNH–CO–$CH_2CH_2$–OH), XVI (X=NHNH–CO–CH(OH)–$CH_3$), XVII (X=NHNH–CO–$OCH_2CH_3$), XVIII (X=NHNH–CO–$CH_2CH_2Cl$), XIX (X=NHNH–CO–$CH_2SCH_3$), XX (X=NHNH–CO–$CH_2SOCH_3$), XXI (X=NHNH–CO–$CH_2CH_2$–$NH_2$), XXII (X=NHNH–CO–$CH_2$–$CH_2$–$COCH_3$), XXIII (X=NHNH–CO–$CH_2$–$NHCOCH_3$), XXIV (X=NHNH–CO–CH=CH–$CH_3$), XXV (X=NHNH–CO–C($CH_3$)$_3$), XXVI (X=NHNH–CO–$CH_2$–$C_6H_5$), XXVII (X=

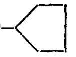
TABLE I. Physicochemical Properties of Intermediates, Z-Phe-X

| Compound No. | X | mp (°C) | [α]D (temp., conc., solvent) | TLC[a] $Rf^1$ | Formula | Analysis (%) Found (Calcd) | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | C | H | N |
| 4 | $NHNH_2$ | 159—160 | −9.8°<br>(21, 0.80, DMF) | 0.62 | $C_{17}H_{19}N_3O_3$ | 64.92<br>(65.16 | 5.94<br>6.11 | 13.27<br>13.41) |
| 5 | $NHNHCOCH_2CH_2CH_3$ | 150—156 | −27.2°<br>(23, 0.50, MeOH) | 0.71 | $C_{21}H_{25}N_3O_4$ | 65.46<br>(65.78 | 6.48<br>6.57 | 10.87<br>10.96) |
| 6 | NHNH-Boc | 107—109 | −23.4°<br>(19, 0.50, MeOH) | 0.71 | $C_{22}H_{27}N_3O_5$ | 64.18<br>(63.90 | 6.61<br>6.58 | 9.88<br>10.16) |
| 7 | $NHNH\text{-}CH_3$ | 118—120 | −13.4°<br>(22, 0.50, DMF) | 0.59 | $C_{18}H_{21}N_3O_3$ | 66.22<br>(66.03 | 6.65<br>6.47 | 12.77<br>12.84) |
| 8 | NHNH- | 160—161 | −22.4°<br>(25, 0.50, MeOH) | 0.66 | $C_{23}H_{23}N_3O_3$ | 70.96<br>(70.93 | 5.90<br>5.95 | 10.65<br>10.79) |
| 9 | NHNH- | 160—161 | −10.0°<br>(22, 0.50, DMF) | 0.67 | $C_{23}H_{29}N_3O_3$ | 69.68<br>(69.85 | 7.62<br>7.39 | 10.56<br>10.63) |
| 10 | $NHN(CH_3)$— | 138—140 | +2.2°<br>(23, 0.50, MeOH) | 0.65 | $C_{24}H_{25}N_3O_3$ | 71.55<br>(71.44 | 6.14<br>6.25 | 10.38<br>10.42) |
| 11 | $NHN(CH_3)_2$ | 122—123 | +2.8°<br>(22, 0.50, MeOH) | 0.64 | $C_{19}H_{23}N_3O_3$ | 67.06<br>(66.84 | 6.83<br>6.79 | 11.83<br>12.31) |
| 12 | $NHNH\text{-}CO\text{-}CH_3$ | 205—206 | −16.4°<br>(23, 0.50, DMF) | 0.65 | $C_{19}H_{21}N_3O_4$ | 64.40<br>(64.21 | 6.00<br>5.96 | 11.59<br>11.83) |
| 13 | $NHNH\text{-}CO\text{-}CH_2CH_2OH$ | 153—154 | −16.7°<br>(27, 0.40, DMF) | 0.37 | $C_{20}H_{23}N_3O_5$ | 62.29<br>(62.32 | 6.01<br>6.02 | 10.62<br>10.90) |
| 14 | $NHNH\text{-}COOCH_2CH_3$ | 134 | −20.6°<br>(23, 0.50, DMF) | 0.69 | $C_{20}H_{23}N_3O_5$ | 62.38<br>(62.32 | 6.09<br>6.02 | 10.49<br>10.90) |
| 15 | $NHNH\text{-}COCH_2SOCH_3$[b] | 139—140 | −2.6°<br>(21, 0.50, DMF) | 0.40 | $C_{17}H_{25}N_3O_5S\cdot 1/2H_2O$ | 51.59<br>(52.01 | 6.76<br>6.67 | 10.79[c]<br>10.70) |
| 16 |  $NHN\begin{matrix}COCH_3\\CH_2CH_2OCOCH_3\end{matrix}$ | 119—120 | −16.1°<br>(23, 0.41, DMF) | 0.64 | $C_{23}H_{27}N_3O_6$ | 62.94<br>(62.57 | 6.39<br>6.16 | 9.65<br>9.52) |

a) See "Experimental." b) Boc-Phe-NHNH-$COCH_2SOCH_3$. c) S, Found 8.25; Calcd 8.17.

Table II. Physicochemical Properties of Intermediates, Z–Tyr–D-Ala–Gly–Phe–X

| Compound No. | X | mp (°C) | [α]D in DMF (Temp., conc.) | TLC[a] $Rf^1$ | Formula | Analysis (%) Found (Calcd) C | H | N |
|---|---|---|---|---|---|---|---|---|
| **17** | NHNH-Boc | 143—146 (dec.) | −25.4° (22, 0.50) | 0.38 | $C_{36}H_{44}N_6O_9$ | 61.05 (61.35 | 6.42 6.29 | 11.58 11.93) |
| **18** | $NHNH_2$ | 182—184 | −23.0° (22, 0.50) | 0.17 | $C_{31}H_{36}N_6O_7 \cdot H_2O$ | 59.89 (59.80 | 6.05 6.14 | 13.29 13.49) |
| **19** | $NHNH-CO-CH_2CH_3$ | 190—191 | −20.5° (23, 0.30) | 0.26 | $C_{34}H_{40}N_6O_8 \cdot 3H_2O$ | 57.24 (57.14 | 5.95 6.48 | 11.34 11.75) |
| **20** | $NHNH-CO-CH_2CH_2CH_3$ | 203—206 | −1.4°[b] (23, 0.50) | 0.28 | $C_{35}H_{42}N_6O_8 \cdot 1/2H_2O$ | 61.05 (61.48 | 6.21 6.33 | 11.95 12.29) |
| **21** | $NHNH-CH_3$ | 184—185 | −27.8° (22, 0.50) | 0.36 | $C_{32}H_{38}N_6O_7$ | 62.03 (62.12 | 6.82 6.19 | 12.84 13.34) |
| **22** |  | 164—165 | −32.6° (19, 0.50) | 0.46 | $C_{37}H_{40}N_6O_7 \cdot 1/2H_2O$ | 64.45 (64.42 | 6.11 5.99 | 11.38 12.18) |
| **23** |  | 181—182 | −34.6° (22, 0.50) | 0.44 | $C_{37}H_{46}N_6O_7 \cdot 1/2H_2O$ | 63.98 (63.86 | 6.64 6.81 | 12.09 12.07) |
| **24** |  | 203—206 | +4.8°[b] (23, 0.50) | 0.43 | $C_{38}H_{42}N_6O_7$ | 65.37 (65.69 | 6.46 6.09 | 11.80 12.10) |
| **25** | $NHN(CH_3)_2$ | 141—142 | −18.7° (25, 0.46) | 0.35 | $C_{33}H_{40}N_6O_7 \cdot H_2O$ | 61.28 (60.90 | 6.29 6.50 | 12.44 12.92) |
| **26** | $NHN-CO-CH_3$ | 210—211 | −22.9° (23, 0.31) | 0.20 | $C_{33}H_{38}N_6O_8 \cdot H_2O$ | 59.99 (59.63 | 6.28 6.07 | 12.62 12.64) |
| **27** | $NHNH-CO-CH_2CH_2-OH$ | 177—178 | −18.1° (27, 0.37) | 0.10 | $C_{34}H_{40}N_6O_9 \cdot H_2O$ | 59.01 (58.78 | 5.96 6.09 | 11.91 12.10) |
| **28** | $NHNH-COOCH_2CH_3$ | 155—156 | −24.2° (23, 0.45) | 0.40 | $C_{34}H_{40}N_6O_9$ | 60.37 (60.34 | 6.33 5.96 | 12.30 12.42) |
| **29** | $NHNH-CO-CH_2SOCH_3$ | 153—154 | −1.8° (21, 0.50) | 0.15 | $C_{34}H_{40}N_6O_9S$ | 57.36 (57.61 | 5.55 5.69 | 11.58[c] 11.85) |
| **30** | $NHN-CO-CH_3$<br>$\diagdown CH_2CH_2OH$ | 129—131 | −25.0° (23, 0.30) | 0.24 | $C_{35}H_{42}N_6O_9$ | 59.55 (59.31 | 6.55 6.25 | 11.37 11.86) |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **31** | NHN-CO-CH$_3$<br>\CH$_2$CH$_2$OCOCH$_3$ | 120—121 | −7.4°<br>(23, 0.32) | 0.38 | $C_{37}H_{44}N_6O_{10}$ | 60.72<br>(60.64 | 6.56<br>6.05 | 10.95<br>11.47) |
| **32** | NHNH-CO-(CH$_2$)$_3$CH$_3$ | 178—179 | −22.4°<br>(23, 0.17) | 0.46 | $C_{36}H_{44}N_6O_8$ | 62.71<br>(62.77 | 6.77<br>6.44 | 11.98<br>12.20) |
| **33** | NHNH-CO-(CH$_2$)$_4$CH$_3$ | 184—185 | −22.7°<br>(23, 0.41) | 0.48 | $C_{37}H_{46}N_6O_8$ | 63.02<br>(63.23 | 7.02<br>6.60 | 11.80<br>11.96) |
| **34** | NHNH-CO-(CH$_2$)$_5$CH$_3$ | 183—184 | −21.1°<br>(25, 0.32) | 0.49 | $C_{38}H_{48}N_6O_8$ | 63.47<br>(63.67 | 6.90<br>6.75 | 11.60<br>11.73) |
| **35** | NHNH-COCH(CH$_3$)$_2$ | 206—207 | −22.3°<br>(24, 0.48) | 0.40 | $C_{35}H_{42}N_6O_8 \cdot H_2O$ | 60.85<br>(60.68 | 6.15<br>6.39 | 11.81<br>12.13) |
| **36** | NHNH-CO-CH$_2$CH(CH$_3$)$_2$ | 228—230 | −23.9°<br>(23, 0.28) | 0.46 | $C_{36}H_{44}N_6O_8 \cdot 1/2H_2O$ | 61.88<br>(61.96 | 6.43<br>6.49 | 12.05<br>12.05) |
| **37** | NHNH-CO-CH(OH)CH$_3$ | 134—136 | −21.7°<br>(24, 0.72) | 0.12 | $C_{34}H_{40}N_6O_9 \cdot 1/2H_2O$ | 59.74<br>(59.55 | 6.29<br>6.02 | 12.47<br>12.25) |
| **38** | NHNH-CO-CH$_2$CH$_2$Cl | 169—170 | −21.1°<br>(23, 0.37) | 0.39 | $C_{34}H_{39}ClN_6O_8$ | 59.08<br>(58.74 | 5.78<br>5.65 | 12.60[d]<br>12.09 |
| **39** | NHNH-CO-CH$_2$CH$_2$-NH-Z | 231—233 | −21.5°<br>(23, 0.26) | 0.26 | $C_{42}H_{47}N_7O_{10} \cdot 1/2H_2O$ | 61.47<br>(61.60 | 5.90<br>5.91 | 11.78<br>11.97) |
| **40** | NHNH-CO-CH$_2$CH$_2$COCH$_3$ | 138—140 | −18.9°<br>(24, 0.27) | 0.28 | $C_{36}H_{42}N_6O_9 \cdot H_2O$ | 60.28<br>(59.99 | 6.04<br>6.15 | 11.46<br>11.66) |
| **41** | NHNH-CO-CH$_2$NH-COCH$_3$ | 171—172 | −20.0°<br>(24, 0.33) | 0.11 | $C_{35}H_{41}N_7O_9$ | 59.67<br>(59.73 | 6.15<br>5.87 | 13.76<br>13.93) |
| **42** | NHNH-CO-CH=CH-CH$_3$[e] | 176—178 | −10.6°<br>(25, 0.43) | 0.35 | $C_{32}H_{42}N_6O_8$ | 60.27<br>(60.17 | 6.88<br>6.63 | 13.29<br>13.13) |
| **43** | NHNH-CO-C(CH$_3$)$_3$ | 156—157 | −24.5°<br>(21, 0.42) | 0.40 | $C_{36}H_{44}N_6O_8$ | 62.66<br>(62.77 | 6.81<br>6.44 | 12.31<br>12.20) |
| **44** | NHNH-COCH$_2$- | 222—223 | −19.6°<br>(24, 0.46) | 0.45 | $C_{39}H_{42}N_6O_8$ | 64.42<br>(64.80 | 6.13<br>5.86 | 11.47<br>11.63) |
| **45** | NHNH-CO--CH$_3$ | 184—186 | −23.3°<br>(24, 0.36) | 0.45 | $C_{39}H_{42}N_6O_8 \cdot H_2O$ | 63.16<br>(63.23 | 5.92<br>5.98 | 11.07<br>11.34) |
| **46** | NHNH-CO- | 228—230 | −26.2°<br>(25, 0.42) | 0.50 | $C_{37}H_{44}N_6O_8 \cdot H_2O$ | 61.42<br>(61.82 | 6.12<br>6.45 | 11.65<br>11.69) |

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*a*) See "Experimental." *b*) MeOH. *c*) S, Found 4.00; Calcd 4.52. *d*) Cl, Found 4.75; Calcd 5.10. *e*) Boc-Tyr-D-Ala-Gly-Phe-NHNH-CO-CH=CH-CH$_3$; This compound was synthesized in a similar manner to that described for the synthesis of **19**, using Boc-Tyr-D-Ala-Gly-Phe-NHNH$_2$ [mp 183—184°C, $[\alpha]_D^{25}$ −20.4° ($c$=0.52, DMF)].

NHNH-CO-$C_6H_4$-$CH_3$), XXVIII (X=NHNH-CO-$C_5H_9$), XXIX (X=NHN($COCH_3$)-$CH_2$-$CH_2$-OH), XXX (X=NHN($COCH_3$)-$CH_2CH_2$-$OCOCH_3$). The analgesic activities of these analogs were compared with that of D-Ala²–Met–enkephalin,[8] H–Tyr–D-Ala–Gly–Phe–$NH_2$[6] or H–Tyr–D-Ala–Gly–Phe–$NHCH_2CH_2CH_2CH_2CH_3$.[9]

Tetrapeptide analogs were synthesized by one of the following two routes. Route A: The key intermediate, Z–Tyr–D-Ala–Gly–OH (3) was prepared by the conventional solution method and condensed with H–Phe–NHN($R_1$)–$R_2$ ($R_1$, $R_2$=H, alkyl or acyl) by the HONB–DCC method.[10] The resulting protected peptide was deblocked by hydrogenation over Pd-black as a catalyst or by treatment with TFA or methanesulfonic acid[11] to obtain the crude peptide, which was purified by column chromatography on Sephadex LH-20. Route B: Z–Tyr–D-Ala–Gly–Phe–$NHNH_2$ (**18**),[12] which was prepared by coupling Z–Tyr–D-Ala–Gly–OH with H–Phe–NHNH–Boc followed by treatment with TFA, was acylated with $R_3$–COOH by the HOBT–DCC method[13] to give Z–Tyr–D-Ala–Gly–Phe–NHNH–CO–$R_3$. The desired peptide was obtained by deblocking and subsequent purification in the same way as in route A. All the analogs obtained were chromatographically pure and gave the expected amino acid ratios. The physicochemical properties of Z–Phe–NHN($R_1$)–$R_2$, the protected tetrapeptides and the analogs are listed in Tables I, II and III, respectively.

Analgesic activities of synthetic analogs were assayed in mice by means of the hot-plate test.[14] Groups of 10 male Ta–$CF_1$ mice weighing 18—24 g were used. Mice were placed

Table III. Physicochemical Properties of Synthetic Analogs

| Analog No. | $[\alpha]_D$ in MeOH (temp., conc.) | TLC[a] | | | Amino acid analysis (recovery %) | | | |
|---|---|---|---|---|---|---|---|---|
| | | $Rf^2$ | $Rf^3$ | $Rf^4$ | Gly | Ala | Tyr | Phe |
| I | +20.5°(22, 0.20) | 0.09 | 0.24 | 0.55 | 1.00 | 0.97 | 0.87 | 0.92(91) |
| II | +19.6°(22, 0.28) | 0.12 | 0.30 | 0.56 | 1.00 | 0.98 | 0.15 | 0.95(82)[b] |
| III | +6.9°(19, 0.28) | 0.25 | 0.51 | 0.74 | 1.00 | 0.98 | 0.94 | 1.06(81) |
| IV | +3.8°(22, 0.27) | 0.21 | 0.44 | 0.63 | 1.00 | 0.96 | 0.97 | 0.99(80) |
| V | +26.2°(23, 0.29) | 0.26 | 0.52 | 0.75 | 1.00 | 1.00 | 0.51 | 0.74(82)[b] |
| VI | +31.2°(25, 0.30) | 0.18 | 0.32 | 0.58 | 1.00 | 0.99 | 0.05 | 0.91(89)[b] |
| VII | +21.6°(23, 0.30) | 0.09 | 0.25 | 0.56 | 1.00 | 0.90 | 0.95 | 0.97(82) |
| VIII | +25.5°(23, 0.23) | 0.14 | 0.30 | 0.59 | 1.00 | 0.99 | 0.96 | 0.94(81) |
| IX | +23.2°(23, 0.55) | 0.18 | 0.41 | 0.64 | 1.06 | 1.00 | 0.93 | 0.93(90) |
| X | +23.5°(23, 0.20) | 0.23 | 0.47 | 0.74 | 1.00 | 1.00 | 0.89 | 1.00(77) |
| XI | +17.9°(23, 0.28) | 0.26 | 0.58 | 0.75 | 1.00 | 1.08 | 1.06 | 1.11(84) |
| XII | +21.3°(25, 0.45) | 0.28 | 0.60 | 0.76 | 1.00 | 0.94 | 0.90 | 0.95(86) |
| XIII | +19.7°(24, 0.28) | 0.24 | 0.40 | 0.68 | 1.00 | 1.00 | 1.02 | 1.07(78) |
| XIV | +21.1°(23, 0.19) | 0.23 | 0.49 | 0.73 | 1.00 | 0.98 | 1.03 | 1.05(82) |
| XV | +20.5°(27, 0.40) | — | 0.29 | 0.60 | 1.00 | 0.97 | 0.83 | 1.00(80) |
| XVI | +16.7°(24, 0.45) | — | 0.30 | 0.55 | 1.00 | 1.10 | 1.07 | 1.12(81) |
| XVII | +18.1°(23, 0.20) | 0.20 | 0.55 | 0.65 | 1.00 | 0.96 | 0.92 | 0.96(82) |
| XVIII | +20.5°(23, 0.20) | 0.20 | 0.48 | 0.65 | 1.00 | 0.99 | 0.92 | 1.01(79) |
| XIX | +18.1°(21, 0.31) | 0.29 | 0.50 | 0.68 | 1.00 | 0.99 | 0.48 | 0.94(81)[b] |
| XX | +18.2°(21, 0.28) | 0.06 | 0.19 | 0.46 | 1.00 | 1.00 | 0.11 | 0.90(90)[b] |
| XXI | +16.7°(23, 0.21) | — | 0.07 | 0.52 | 1.00 | 1.02 | 0.97 | 1.00(85)[c] |
| XXII | +8.5°(24, 0.30) | 0.16 | 0.39 | 0.55 | 1.00 | 0.98 | 0.97 | 1.01(91) |
| XXIII | +14.4°(24, 0.30) | 0.04 | 0.24 | 0.56 | 2.00 | 1.03 | 0.96 | 0.97(87) |
| XXIV | +14.5°(25, 0.20) | 0.24 | 0.41 | 0.66 | 1.00 | 0.98 | 0.97 | 1.06(86) |
| XXV | +19.2°(21, 0.27) | 0.40 | 0.45 | 0.71 | 1.00 | 0.88 | 1.08 | 1.18(79) |
| XXVI | +18.5°(24, 0.28) | 0.29 | 0.56 | 0.77 | 1.00 | 0.98 | 0.95 | 0.97(79) |
| XXVII | +3.1°(24, 0.32) | 0.30 | 0.58 | 0.78 | 1.00 | 0.94 | 0.92 | 0.94(80) |
| XXVIII | +20.0°(25, 0.23) | 0.28 | 0.54 | 0.70 | 1.00 | 1.08 | 1.09 | 1.15(88) |
| XXIX | +15.3°(24, 0.43) | 0.19 | 0.36 | 0.60 | 1.00 | 0.87 | 0.90 | 1.00(88) |
| XXX | +18.1°(23, 0.31) | 0.22 | 0.42 | 0.66 | 1.00 | 0.88 | 0.92 | 0.98(83) |

*a*) See "Experimental." *b*) See ref. 16. *c*) β-Ala: 1.15.

on a hot plate at 55°C, and licking of a hind foot or jumping was observed. Animals showing such responses within 20 seconds were selected, and test compounds were administered subcutaneously or intravenously. The analgesic activities of analogs were evaluated by comparing the latency periods of treated animals and control animals. Details of assays will be described elsewhere.[15] The potencies of analogs relative to that of morphine hydrochloride are summarized in Table IV.

TABLE IV. Analgesic Activities of H–Tyr–D-Ala–Gly–Phe–X

| Analog No. | X | Relative potency[a)] | |
|---|---|---|---|
| | | *i.v.* | *s.c.* |
| I | $NHNH_2$ | $<0.25$ | 0.1 |
| II | $NHNH-CH_3$ | | 0.025 |
| III | $NHNH-C_6H_5$ | 0.05 | |
| IV | $NHNH-C_6H_{11}$ | $<0.05$ | |
| V | $NHN(CH_3)-C_6H_5$ | 0.01 | |
| VI | $NHN(CH_3)_2$ | | 0.05 |
| VII | $NHNH-COCH_3$ | 0.5 | 0.5 |
| VIII | $NHNH-COCH_2CH_3$ | 0.5 | 0.5 |
| IX | $NHNH-CO(CH_2)_2CH_3$ | 0.5 | 0.5 |
| X | $NHNH-CO(CH_2)_3CH_3$ | 0.5 | 0.5 |
| XI | $NHNH-CO(CH_2)_4CH_3$ | 0.5 | $<0.25$ |
| XII | $NHNH-CO(CH_2)_5CH_3$ | | 0.1 |
| XIII | $NHNH-COCH(CH_3)_2$ | 0.5 | 0.5 |
| XIV | $NHNH-COCH_2CH(CH_3)_2$ | 0.5 | $<0.5$ |
| XV | $NHNH-COCH_2CH_2-OH$ | | 1.0 |
| XVI | $NHNH-COCH(OH)CH_3$ | 0.5 | $<0.5$ |
| XVII | $NHNH-CO-OCH_2CH_3$ | 0.5 | 0.5 |
| XVIII | $NHNH-COCH_2CH_2-Cl$ | 0.5 | 0.5 |
| XIX | $NHNH-COCH_2SCH_3$ | | 0.5 |
| XX | $NHNH-COCH_2SOCH_3$ | | 0.5 |
| XXI | $NHNH-COCH_2CH_2-NH_2$ | 0.1 | |
| XXII | $NHNH-COCH_2CH_2-COCH_3$ | | 0.5 |
| XXIII | $NHNH-COCH_2-NHCOCH_3$ | 0.5 | |
| XXIV | $NHNH-COCH{=}CH-CH_3$ | | 0.1 |
| XXV | $NHNH-COC(CH_3)_3$ | | 0.25 |
| XXVI | $NHNH-COCH_2C_6H_5$ | 0.1 | |
| XXVII | $NHNH-COC_6H_4-CH_3$ | 0.1 | |
| XXVIII | $NHNH-COC_5H_9$ | | 0.1 |
| XXIX | $NHN(COCH_3)-CH_2CH_2OH$ | | 0.5 |
| XXX | $NHN(COCH_3)-CH_2CH_2OCOCH_3$ | | 0.25 |
| | D-Ala$^2$–Met–enkephalin[b)] | $<0.025$ | |
| | H–Tyr–D-Ala–Gly–Phe–$NH_2$[c)] | 0.05 | 0.05 |
| | H–Tyr–D-Ala–Gly–Phe–$NH-CH_2CH_2CH_2CH_2CH_3$[d)] | | $<0.05$ |

a) Morphine=1. Minimum effective dose of morphine·HCl=0.5 mg/kg (weight/weight). b) See ref. 8. c) See ref. 6. d) See ref. 9.

As shown in Table IV, the tetrapeptide hydrazide, H–Tyr–D-Ala–Gly–Phe–$NHNH_2$ (I), caused profound analgesia after subcutaneous injection compared to D-Ala$^2$–Met–enkephalin,[8] and was twice as potent as the tetrapeptide amide analog H–Tyr–D-Ala–Gly–Phe–$NH_2$.[6] Alkylation of the hydrazide group decreased the potency. In contrast, acylation of the hydrazide group of the analog I resulted in a remarkable increase in activity. For instance, the analog IX was found to be approximately half as potent as morphine following intravenous or subcutaneous administration. Thus, the C-terminal Met residue of D-Ala$^2$–Met–enkephalin is unnecessary for *in vivo* biological activity, and the tetrapeptide analog, when the C-terminal carboxyl group is suitably blocked, is superior to a pentapeptide analog such as D-Ala$^2$–Met–

enkephalin.

The result that H–Tyr–D-Ala–Gly–Phe–NHNH–CO–$CH_2CH_2CH_3$ (IX) was more than ten times as potent as H–Tyr–D-Ala–Gly–Phe–NH–$CH_2CH_2CH_2CH_2CH_3$,[9] indicates the importance of the acyl-hydrazide part of the analog IX for potent activity.

The data in Table IV also show that among the acyl-hydrazide analogs those with a lower straight chain acyl-hydrazide are the most active, even if one hydrogen of the acyl group is replaced by –OH, –Cl, –$OCH_3$, –S–$CH_3$ or –SO–$CH_3$, and that introduction of a long chain acyl or a bulky acyl group reduces the biological activity. This suggests that the size of the acyl group as well as the basicity of the hydrazide part is critical, and that the acyl-hydrazide part of this new type of enkephalin analog might make a significant contribution to its binding to opiate receptors, or at least play an important role in a peptide-receptor interaction.

## Experimental

Melting point determinations were performed with a Yamato Model MP-21 melting point apparatus and are uncorrected. Evaporations were carried out with a rotary evaporator. Rotations were determined with a Perkin-Elmer Model 141 polarimeter. Amino acid analyses were run on acid hydrolysates prepared in 6 N HCl at 110°C for 24 h. The analyses were performed on a Hitachi KLA-3B amino acid analyzer. The purity of intermediates and end products was checked by thin layer chromatography on silica gel (precoated silica gel plate 60 $F_{254}$, Merck). The solvents employed were: $Rf^1$, $CHCl_3$–MeOH–AcOH (9: 1: 0.5); $Rf^2$, $CHCl_3$–MeOH–AcOH (8: 2: 0.5); $Rf^3$, AcOEt–pyridine–AcOH–$H_2O$ (60: 20: 6: 11); $Rf^4$, *n*-butanol–AcOH–$H_2O$ (4: 1: 1); $Rf^5$, AcOEt–*n*-butanol–AcOH–$H_2O$ (1: 1: 1: 1).

**Z-D-Ala-Gly-OEt** (1)——To a solution of Z–D-Ala–OH (8.9 g) and H–Gly–OEt·HCl (5.9 g) in THF (100 ml) were added TEA (5.9 ml), HONB (7.9 g) and DCC (9.1 g) at 0°C, and the mixture was stirred at 0°C for 4 h. After being stirred for an additional 10 h at room temperature the reaction mixture was filtered to remove the DC-urea precipitate and the filtrate was evaporated to dryness. The residue was extracted with AcOEt (100 ml) and the solution was treated in the usual manner (washed with 0.1 N HCl, 5% $NaHCO_3$ and $H_2O$, dried over anhydr. $Na_2SO_4$ and evaporated to dryness). The resulting crystals were collected by filtration and recrystallized from AcOEt–pet. ether; yield, 10.0 g (81%), mp 97°C, $[\alpha]^{23}_{D}$ $+1.7°$ ($c=1.0$, DMF), $Rf^1=0.65$. *Anal.* Calcd for $C_{15}H_{20}N_2O_5$: C, 58.43; H, 6.54; N, 9.09. Found: C, 58.13; H, 6.77; N, 9.31.

**Z-Tyr-D-Ala-Gly-OEt** (2)——The compound **1** (8.4 g) was hydrogenated over Pd-black catalyst in EtOH (50 ml). The solution was filtered to remove the catalyst and the filtrate was evaporated to dryness. The residue was dissolved in THF (100 ml), and Z–Tyr–ONB (12.8 g) was added to the solution. After the mixture had been stirred for 12 h at room temperature, the solvent was evaporated off. The resulting residue was extracted with AcOEt (200 ml) and the extract was treated in the usual manner. The resulting crystals were collected by filtration and recrystallized from AcOEt–pet. ether; yield, 10.6 g (84%), mp 171—172°C, $[\alpha]^{24}_{D}$ $-12.2°$ ($c=0.50$, DMF), $Rf^1=0.67$. *Anal.* Calcd for $C_{24}H_{29}N_3O_7$: C, 61.13; H, 6.20; N, 8.91. Found: C, 61.23; H, 6.27; N, 8.77.

**Z-Tyr-D-Ala-Gly-OH** (3)——A solution of the compound **2** (9.9 g) in MeOH (20 ml) was treated with 1 N NaOH (42 ml) at 0°C. After being stirred at room temperature for 1 h, the solution was neutralized by addition of 1 N HCl (42 ml) and evaporated to dryness. The resulting crystals were collected by filtration, washed with $H_2O$, and dried; yield, 8.4 g (90%), mp 124°C, $[\alpha]^{25}_{D}$ $-16.7°$ ($c=0.54$, DMF), $Rf^1=0.08$. *Anal.* Calcd for $C_{22}H_{25}N_3O_7 \cdot H_2O$: C, 57.27; H, 5.89; N, 9.10. Found: C, 57.40; H, 5.84; N, 8.97.

**Z-Phe-NHNH$_2$** (4)———Z–Phe–OH (59.0 g) was dissolved in MeOH (200 ml), then 6 N HCl–dioxane (5 ml) was added, and the mixture was allowed to stand overnight at room temperature. The MeOH was evaporated and the resulting residue was dissolved in ether (300 ml). The solution was washed with water and dried over anhydr. $Na_2SO_4$. The ether was evaporated and the resulting methyl ester was dissolved in MeOH (150 ml). Hydrazine hydrate (16 ml) was added to the solution, which was allowed to stand at room temperature for 2 days. The MeOH was evaporated and the resulting crystals were collected by filtration and recrystallized from MeOH; yield, 49.0 g (79%), mp 159—160°C, $[\alpha]^{21}_{D}$ $-9.8°$ ($c=0.80$, DMF), $Rf^1=0.62$. *Anal.* Calcd for $C_{17}H_{19}N_3O_3$: C, 65.16; H, 6.11; N, 13.41. Found: C, 64.92; H, 5.94; N, 13.27.

**Z-Phe-NHNH-CO-$CH_2CH_2CH_3$** (5)——$CH_3CH_2CH_2COCl$ (0.9 ml) and TEA (1.1 ml) were added to a solution of the compound **4** (2.5 g) in DMF (30 ml) under cooling. After being stirred at room temperature for 5 h, the mixture was poured into $H_2O$ (100 ml). The resulting crystals were collected by filtration, washed with $H_2O$ and dried; yield, 2.6 g (85%), mp 150—156°C, $[\alpha]^{23}_{D}$ $-27.2°$ ($c=0.50$, MeOH), $Rf^1=0.71$. *Anal.* Calcd for $C_{21}H_{25}N_3O_4$: C, 65.78; H, 6.57; N, 10.96. Found: C, 65.46; H, 6.48; N, 10.87.

**Z-Phe-NHNH-Boc** (6)[17]——A mixture of Z–Phe–ONB[10] (18.4 g) and $NH_2NH$–Boc (5.3 g) in THF (100 ml) was stirred at room temperature overnight then evaporated to dryness. The resulting residue was extracted with AcOEt (150 ml) and treated in the usual manner. The resulting residue was crystallized with pet. ether, collected by filtration and purified by recrystallization from AcOEt–pet. ether; yield, 13.2 g (80%), mp 107—109°C, $[\alpha]_D^{19}$ −23.4° ($c$=0.50, MeOH), $Rf^1$=0.71. *Anal.* Calcd for $C_{22}H_{27}N_3O_5$: C, 63.90; H, 6.58; N, 10.16. Found: C, 64.18; H, 6.61; N, 9.88.

**Z-Tyr-D-Ala-Gly-Phe-NHNH-Boc (17)**——The compound **6** (3.6 g) was dissolved in MeOH (100 ml) and hydrogenated over Pd-black. The catalyst was filtered off, the filtrate was evaporated to dryness. The residue was dissolved in DMF (10 ml). On the other hand, a mixture of **3** (3.5 g) and HONB (1.6 g) in THF (50 ml) was cooled to 0°C, and DCC (1.8 g) was added. After being stirred at 0°C for 6 h, the mixture was filtered to remove the insolubles. The filtrate was combined with the amine component prepared above, and the mixture was stirred at room temperature overnight. The solvent was evaporated and the residue was extracted with AcOEt (150 ml). The AcOEt solution was treated in the usual manner, and the resulting residue was crystallized from pet. ether; yield, 3.7 g (67%), mp 143—146°C (dec.), $[\alpha]_D^{22}$ −25.4° ($c$=0.50, DMF), $Rf^1$=0.38. *Anal.* Calcd for $C_{36}H_{44}N_6O_9$: C, 61.35; H, 6.29; N, 11.93. Found: C, 61.05; H, 6.42; N, 11.58.

**Z-Tyr-D-Ala-Gly-Phe-NHNH$_2$ (18)**——The compound **17** (3.5 g) was dissolved in TFA (30 ml) and the solution was allowed to stand at room temperature for 20 min. The TFA was evaporated and the residue was treated with ether, and collected by filtration. The powder obtained was dried and dissolved in DMF (10 ml), then TEA (1.4 ml) was added. The DMF was evaporated and the residue was treated with ether, collected by filtration and reprecipitated from MeOH–ether; yield, 2.8 g (96%), mp 182—184°C, $[\alpha]_D^{22}$ −23.0° ($c$=0.50, DMF), $Rf^1$=0.17. *Anal.* Calcd for $C_{31}H_{36}N_6O_7 \cdot H_2O$: C, 59.80; H, 6.14; N, 13.49. Found: C, 59.89; H, 6.05; N, 13.29.

**Z-Tyr-D-Ala-Gly-Phe-NHNH-CO-CH$_2$CH$_3$ (19)**——DCC (250 mg) was added to a mixture of **18** (495 mg), HOBT (220 mg) and propionic acid (0.1 ml) in DMF (10 ml) at 0°C. The mixture was stirred at 0°C for 4 h then at room temperature overnight. The formed DC-urea precipitate was filtered off, and the filtrate was evaporated to dryness. The residue was extracted with AcOEt (100 ml) and the AcOEt solution was treated in the usual manner. The resulting residue was treated with ether, collected by filtration and crystallized from $CH_3CN$; yield, 0.48 g (70%), mp 190—191°C, $[\alpha]_D^{23}$ −20.5° ($c$=0.30, DMF), $Rf^1$ 0.26. *Anal.* Calcd for $C_{34}H_{40}N_6O_8 \cdot 3H_2O$: C, 57.14; H, 6.48; N, 11.75. Found: C, 57.24; H, 5.95; N, 11.34.

**Z-Tyr-D-Ala-Gly-Phe-NHNH-CO-CH$_2$CH$_2$CH$_3$ (20)**——The compound **5** (0.50 g) was dissolved in MeOH (50 ml) and catalytic reduction was carried out with Pd-black catalyst. The mixture was filtered to remove the catalyst and the filtrate was evaporated to dryness. The residue was dissolved in THF (20 ml). On the other hand, **3** (0.53 g) and HONB (0.24 g) were dissolved in THF (30 ml), then DCC (0.27 g) was added under cooling. After the mixture had been stirred at 0°C for 4 h, the DC-urea precipitate was filtered off. The filtrate was combined with the amine component prepared above, and the mixture was stirred overnight then evaporated to dryness. The resulting residue was treated with ether, collected by filtration and purified by crystallization from $CH_3CN$; yield, 0.42 g (50%), mp 203—206°C, $[\alpha]_D^{23}$ −1.4° ($c$=0.50, MeOH), $Rf^1$=0.28. *Anal.* Calcd for $C_{35}H_{42}N_6O_8 \cdot 1/2H_2O$: C, 61.48; H, 6.33; N, 12.29. Found: C, 61.05; H, 6.21; N, 11.95.

**Synthesis of Tetrapeptide Analogs of Enkephalin. H-Tyr-D-Ala-Gly-Phe-NHNH-CO-CH$_2$CH$_3$ (VIII)**—— The compound **19** (0.25 g) was dissolved in MeOH (50 ml), then AcOH (1 ml) was added, and catalytic reduction was carried out over Pd-black. The catalyst was filtered off, then the filtrate was evaporated to dryness and the residue was dissolved in a small amount of 1 N aqueous acetic acid. The solution was applied to a column of Sephadex LH-20 (2.5 × 120 cm), which was eluted with 1 N aqueous acetic acid. The fractions from 310 ml through 335 ml were combined and lyophilized; yield, 130 mg, $[\alpha]_D^{23}$ +25.5° ($c$=0.23, MeOH), $Rf^2$=0.14; $Rf^3$=0.30; $Rf^4$=0.59. Amino acid analysis: Gly 1.00; Ala 0.99; Tyr 0.96; Phe 0.94 (average recovery 81%). $^1$H-NMR ($D_2O$), $\delta$: 1.15 (3H, t, CO-CH$_2$C$\underline{H}_3$), 2.36 (2H, q, CO-C$\underline{H}_2$CH$_3$).

**H-Tyr-D-Ala-Gly-Phe-NHNH-CO-CH$_2$CH$_2$CH$_3$ (IX)**——The compound **20** (0.25 g) was dissolved in MeOH (50 ml), then AcOH (0.2 ml) was added. Catalytic hydrogenation was carried out over Pd-black catalyst. The catalyst was filtered off and the filtrate was evaporated to dryness. The residue was dissolved in a small amount of $H_2O$ and applied to a column of Sephadex LH-20 (2.5 × 120 cm). Elution was carried out with 1 N aqueous acetic acid and fractions from 320 ml through 340 ml were collected and lyophilized; yield, 110 mg, $[\alpha]_D^{23}$ +23.2° ($c$=0.55, MeOH), $Rf^2$=0.18; $Rf^3$=0.41; $Rf^4$=0.64. Amino acid analysis: Gly 1.06; Ala 1.00; Tyr 0.93; Phe 0.93 (average recovery 90%). $^1$H-NMR ($D_2O$), $\delta$: 0.95 (3H, t, CO-CH$_2$CH$_2$-C$\underline{H}_3$), 1.65 (2H, sext, CO-CH$_2$C$\underline{H}_2$CH$_3$), 2.30 (2H, t, CO-C$\underline{H}_2$CH$_2$CH$_3$). H-Tyr-D-Ala-Gly-Phe-NHNH-CO-CH$_2$CH$_2$-CH$_3$·HCl was obtained as crystals from $CH_3CN$–EtOH; mp 169—170°C, $[\alpha]_D^{24}$ +25.7° ($c$=0.55, MeOH).

Analogs I, X—XIV, XVI—XVIII, XXI—XXVIII were synthesized in exactly the same way as described for the synthesis of the analog VIII. Analogs II—VII, XV, XX, XXX were synthesized by the same procedure from **3** and the corresponding Z-Phe-NHN(R$_1$)-R$_2$ as described for the synthesis of the analog IX. The analog XIX was obtained by treatment of the analog XX with 4% aqueous thioglycolic acid, and for the synthesis of the analog XXIX, **30** was prepared by saponification of **31** with aqueous sodium hydroxide. Physicochemical properties of Z-Phe-NHN(R$_1$)-R$_2$, the protected tetrapeptides and final products are listed in Tables I, II and III, respectively.

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## References and Notes

1) Amino acids, peptides and their derivatives in this paper are of the L-configuration. The following abbreviations are used: Z=benzyloxycarbonyl, Boc=*tert*-butoxycarbonyl, HONB=N-hydroxy-5-norbornene-2,3-dicarboximide, HOBT=N-hydroxybenzotriazole, DCC=dicyclohexylcarbodiimide, DC-urea=dicyclohexylurea, TFA=trifluoroacetic acid, TEA=triethylamine, DMF=N,N-dimethylformamide, THF=tetrahydrofuran, AcOEt=ethyl acetate, OEt=ethyl ester, Thz=thiazolidine-4-carboxylic acid, Met(O)-ol=methioninol sulfoxide, MePhe=N-methyl-Phe.
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