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Studies on Analgesic Oligopeptides. II.[1,2] Structure-Activity Relationship among Thirty Analogs of a Cyclic Dipeptide, Cyclo(-Tyr-Arg-)

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Thirty diketopiperazines were synthesized as analogs of cyclo(–Tyr–Arg–). The analgesic activities of these analogs were evaluated after intracerebral administration in mice. In the cyclo(–X–Arg–) series of analogs, cyclo[–Tyr(Et)–Arg–] showed the most potent activity. In the cyclo(–Tyr–Y–) series of analogs, the activity decreased in the order Y=homoarginine, *p*-guanidinophenylalanine, 2-amino-4-guanidinobutyric acid, Lys, Orn, His, α,γ-diaminobutyric acid and Pro. Among the analogs synthesized, cyclo[–Tyr(Et)–Har–], which was designed on the basis of the above results, exhibited remarkably potent analgesic activity, being 17 times more potent than cyclo(–Tyr–Arg–) and nearly as potent as morphine on a molar basis. The structure–activity relation of cyclo(–Tyr–Arg–) is discussed in the light of these results.

Keywords——diketopiperazines; cyclo(–Tyr–Arg–) analogs; cyclo[–Tyr(Et)–Har–]; analgesic activity; intracerebral administration in mice; structure–activity relationship

In a previous paper,[1] it was reported that cyclo(–Tyr–Arg–) showed more potent analgesic activity than its three stereoisomers. This paper deals with a further study of the structure and analgesic activity relationship of cyclo(–Tyr–Arg–). First, the Tyr residue in cyclo(–Tyr–Arg–) was replaced by another amino acid or derivative and the analgesic activity of the resulting synthetic diketopiperazines was assayed. That is, X in the general formula cyclo(–X–Arg) was as follows, X=β-cyclohexylalanine (Cha) (**1**), Leu (**2**), Val (**3**), Ser (**4**), Ala (**5**), Phe (**6**), Trp (**7**), 5-hydroxytryptophan (5HTP) (**8**), Tyr(Me) (**9**), Tyr(Et) (**10**), Tyr-(3,5-diBr) (**11**), Trp(CHO) (**12**), MeTyr (**13**) or AlTyr (**14**). As shown in Table I, **8**, **10** and **12** showed more potent analgesic activity than the other synthetic analogs in this series. Second, the Arg residue in cyclo(–Tyr–Arg–) was replaced by another amino acid or derivative and the analgesic activity of the diketopiperazines was assayed. That is, Y in the general formula cyclo(–Tyr–Y–) was as follows, Y=homoarginine (Har) (**15**), *p*-guanidinophenylalanine (Gph) (**16**), 2-amino-4-guanidinobutyric acid (Gbu) (**17**), Lys (**18**), Orn (**19**), α,γ-diaminobutyric acid (Dab) (**20**), His (**21**), Pro (**22**) or Arg(DHCH) (**23**). As shown in Table I, **15** showed the most potent analgesic activity among the synthetic analogs in this series. Finally, amino acid residues which appeared to produce higher activity in the above two series of diketopiperazines were assembled together to form diketopiperazines. Thus, cyclo(–5HTP–Har–) (**24**), cyclo[–Tyr(Et)–Har–] (**25**) and cyclo[–Trp(CHO)–Har–] (**26**) were synthesized. Further, in order to examine the effect of the alkyl group of *O*-alkyl Tyr in detail, the following four analogs were synthesized: cyclo[–Tyr(*n*-Pr)–Har–] (**27**), cyclo[–Tyr(iso-Pr)–Har–] (**28**), cyclo[–Tyr(*n*-Bu)–Har–] (**29**), [–Tyr(*tert*-Bu)–Har–] (**30**). As shown in Table I, **25** showed the most potent analgesic activity among the thirty diketopiperazines tested in this study.

All the analogs were synthesized by a similar route, which is not subject to racemization;[1,3] Boc-dipeptide methyl (ethyl or benzyl) ester was prepared by the DCC or WSCI method in the presence of HOBt[4] or HONB[5] as an additive, and cyclization of the dipeptide ester was carried out by the acetic acid-catalyzed method.[3] Protecting groups for side chain functional groups were as follows, Bzl was used for the hydroxyl group in Tyr and Ser; Z was used for the N^{ω}-amino group is Lys, Orn and Dab; HCl was used for the guanidino group in Arg, Har and Gph.

TABLE I. Analgesic Activities of Diketopiperazines after Intracerebral Administration to Mice

Diketopiperazine	ED_{50} value (nmol/mouse)[a]	Relative potency[b]
C. (-Tyr-Arg-)	33.0(26.9— 40.4)	1
Met-enkephalin	170.0(146.6—197.2)	0.19
Morphine	1.5(1.1— 2.1)	22.0
C. (-Cha-Arg-) (**1**)	37.8(24.4— 58.6)	0.87
C. (-Leu-Arg-) (**2**)	62.0(54.4— 70.7)	0.53
C. (-Val-Arg-) (**3**)	46.0(35.1— 60.3)	0.72
C. (-Ser-Arg-) (**4**)	72.0(51.8—100.0)	0.46
C. (-Ala-Arg-) (**5**)	46.0(36.5— 58.0)	0.72
C. (-Phe-Arg-) (**6**)	47.1(32.0— 69.2)	0.70
C. (-Trp-Arg-) (**7**)	41.0(30.8— 54.5)	0.81
C. (-5HTP-Arg-) (**8**)	11.3(8.3— 15.1)	2.92
C. [-Tyr(Me)-Arg-] (**9**)	18.5(12.5— 27.4)	1.78
C. [Tyr(Et)-Arg-] (**10**)	3.2(2.0— 5.1)	10.31
C. [-Tyr(3,5-diBr)Arg-] (**11**)	12.0(9.8— 14.8)	2.75
C. [-Trp(CHO)-Arg-] (**12**)	8.2(6.7— 10.0)	4.02
C. (-MeTyr-Arg-) (**13**)	23.4(15.5— 35.3)	1.41
C. (-AlTyr-Arg-) (**14**)	24.5(14.0— 42.8)	1.35
C. (-Tyr-Har-) (**15**)	13.2(11.0— 15.8)	2.50
C. (-Tyr-Gph-) (**16**)	17.0(14.0— 20.6)	1.94
C. (-Tyr-Gbu-) (**17**)	23.8(17.9— 31.5)	1.39
C. (-Tyr-Lys-) (**18**)	103.4(74.9—141.6)	0.32
C. (-Tyr-Orn-) (**19**)	180.0(121.6—266.4)	0.18
C. (-Tyr-Dab-) (**20**)	550.0(443.5—682.0)	0.06
C. (-Tyr-His-) (**21**)	260.0(170.4—396.5)	0.13
C. (-Tyr-Pro-) (**22**)	2470.0<	—
C. [-Tyr-Arg(DHCH)-] (**23**)	40.4(31.3— 52.1)	0.82
C. (-5HTP-Har-) (**24**)	13.8(11.9— 16.0)	2.39
C. [-Tyr(Et)-Har-] (**25**)	1.9(1.1— 3.2)	17.37
C. [-Trp(CHO)-Har-] (**26**)	6.2(4.4— 8.7)	5.32
C. [-Tyr(*n*-Pr)-Har-] (**27**)	2.9(2.1— 4.1)	11.38
C. [-Tyr(iso-Pr)-Har-] (**28**)	3.7(2.7— 5.0)	8.92
C. [-Tyr(*n*-Bu)-Har-] (**29**)	4.9(3.3— 7.2)	6.74
C. [-Tyr(*tert*-Bu)-Har-] (**30**)	14.7(12.0— 17.9)	2.54

a) 95% confidence limits are given in parentheses.
b) The value for cyclo(-Tyr-Arg-) was taken as 1.

In order to synthesize **10** and **25**, Boc-Tyr(Et)-OH was prepared by *O*-ethylation of N^{α}-acetyl-Tyr-OH[6] with diethyl sulfate by a method similar to that described for the preparation of H-Tyr(Me)-OH by Siedel *et al.*[6] followed by *tert*-butoxycarbonylation with 2-*tert*-butoxycarbonyloxyimino-2-phenylacetonitrile (Boc-ON).[7] Condensation of Boc-Tyr(Et)-OH and H-Arg(HCl)-OMe[8] or H-Har(HCl)-OMe gave the corresponding dipeptide intermediate, Boc-Tyr(Et)-Arg-OMe or Boc-Tyr(Et)-Har-OMe. For the synthesis of **13**, Boc-MeTyr(Bzl)-OH[9] was prepared from Boc-Tyr(Bzl)-OH with methyl iodide and NaH by the method of Cheung and Benoiton,[9] but this method for the preparation of Boc-AlTyr(Bzl)-OH using allyl bromide and NaH was not successful. Thus, H-AlTyr-OH was prepared by allylation of N^{α}-Tfa-Tyr(Bzl)-OBzl with allyl bromide and powdered KOH using a method similar to that described for *N*-alkylation of amines by Johnstone *et al.*,[10] followed by acid hydrolysis with 6 N HCl to give H-AlTyr-OH as a crystalline material in 40% yield. Subsequent *tert*-butoxycarbonylation with Boc-ON gave Boc-AlTyr-OH as an oil, which was condensed with H-Arg(HCl)-OMe to give a dipeptide intermediate, Boc-AlTyr-Arg-OMe. For the synthesis of **16**, Boc-Phe(NO_2)-OEt was prepared from Boc-Phe(NO_2)-OH[11] and ethyl bromide with the aid of KF,[12] followed by catalytic hydrogenation to give Boc-Phe(NH_2)-OEt. Guanidylation of the compound using 1-amidino-3,5-dimethylpyrazole by a method similar to that

Table II. Yields and Physical Constants of Dipeptide Intermediates

Compound	Yield (%)	mp (°C)	$[\alpha]_D$ [a]	Formula	Analysis (%) [b] Calcd (Found) C	H	N	*Rf* (A) [c]
Boc-Cha-Arg-OMe	68	102—107	−31.0	$C_{21}H_{39}N_5O_5 \cdot CH_3COOH \cdot H_2O$ [d]	53.16 (53.02	8.73 8.72	13.48 13.34)	0.30
H-Leu-Arg-OMe·2HCl	77	120—122	+14.0	$C_{13}H_{27}N_5O_3 \cdot 2HCl \cdot 3/2H_2O$	38.90 (38.69	8.04 8.48	17.67 17.08)	0.25
H-Val-Arg-OMe·2HCl	80	Amorph.	−46.0	$C_{12}H_{25}N_5O_3 \cdot 2HCl \cdot H_2O$	38.10 (38.53	7.72 7.90	18.51 17.88)	0.21
H-Ser(Bzl)-Arg-OMe·2HCl	85	Amorph.	+22.0	$C_{17}H_{27}N_5O_4 \cdot 2HCl$	46.58 (46.82	6.67 6.88	16.12 16.12)	0.35
Boc-Ala-Arg-OMe	53	78— 83 (dec.)	−27.0 ($c=0.4$)	$C_{15}H_{29}N_5O_5 \cdot 3/2\ CH_3COOH$ [d]	48.10 (48.49	7.87 8.41	15.58 15.33)	0.13
Boc-Phe-Arg-OMe	60	100—105	−22.2 ($c=0.9$)	$C_{21}H_{33}N_5O_5$	57.91 (57.81	7.64 7.48	16.08 16.10)	0.34
Boc-Trp-Arg-OMe	78	102—106	−33.7	$C_{23}H_{34}N_6O_5$	58.21 (58.63	7.22 7.12	17.71 17.54)	0.30
Boc-5HTP-Arg-OMe	85	91— 96	−27.3 ($c=0.4$, 1% AcOH)	$C_{23}H_{34}N_6O_6 \cdot 1/2\ HCl$	54.29 (53.93	6.84 7.21	16.52 16.04)	0.29
H-Tyr(Me)-Arg-OMe·2HCl	78	84— 88	+11.0	$C_{17}H_{27}N_5O_4 \cdot 2HCl \cdot H_2O$	44.74 (44.93	6.85 7.24	15.34 15.27)	0.26
Boc-Tyr(Et)-Arg-OMe	58	74— 77	+18.0	$C_{23}H_{37}N_5O_6 \cdot CH_3COOH$ [d]	55.64 (56.03	7.66 8.17	12.98 12.56)	0.29
Boc-Tyr(3,5-diBr)-Arg-OMe	75	188—190 (dec.)	+29.0 ($c=2.0$)	$C_{21}H_{31}N_5O_6Br_2 \cdot 1/2\ HCl$	40.19 (40.61	5.06 5.20	11.16 10.85)	0.33
H-Trp(CHO)-Arg-OMe·2HCl	89	117—121	+17.0	$C_{19}H_{26}N_6O_4 \cdot 2HCl \cdot H_2O$	46.25 (46.62	6.13 6.44	17.03 16.67)	0.25
Boc-MeTyr(Bzl)-Arg-OMe·HCl	91	87— 91	−57.6	$C_{22}H_{35}N_5O_6 \cdot HCl$	58.82 (59.22	7.15 7.50	11.83 11.87)	0.31
Boc-AlTyr-Arg-OMe·HCl	82	94—100	−44.0 ($c=0.7$)	$C_{24}H_{37}N_5O_6 \cdot HCl \cdot 2H_2O$	51.10 (50.84	7.51 7.41	12.42 12.90)	0.37
H-Tyr(Bzl)-Har-OMe·2HCl	62	192—195	+15.0	$C_{24}H_{33}N_5O_4 \cdot 2HCl$	54.53 (54.82	6.68 6.83	13.25 13.17)	0.36
H-Tyr(Bzl)-Gph-OEt·2HCl	51	180—182	+24.0	$C_{28}H_{33}N_5O_4 \cdot 2HCl \cdot H_2O$	56.56 (56.98	6.27 6.25	11.78 11.31)	0.52
Boc-Tyr(Bzl)-Lys(Z)-OMe	69	143—147	−34.0 (DMF)	$C_{36}H_{45}N_3O_8$	66.75 (67.22	7.00 7.42	6.50 7.00)	0.89
Boc-Orn(Z)-Tyr-OBzl	89	95— 96	−27.0	$C_{34}H_{42}N_3O_8 \cdot H_2O$	65.79 (66.08	6.82 7.00	6.77 6.71)	0.73
Boc-Dab(Z)-Tyr-OBzl	72	53— 56	−33.0 (DMF)	$C_{33}H_{39}N_3O_8$	65.44 (65.85	6.49 6.53	6.94 6.70)	0.78
Boc-Tyr(Bzl)-His-OMe [e]								
Boc-Tyr-Pro-OBzl	85	117—119	−54.0	$C_{26}H_{32}N_2O_6$	66.65 (66.08	6.88 6.91	5.98 6.20)	0.57
H-5HTP-Har-OMe·2Tos	43	132—134	+42.4 ($c=0.4$)	$C_{19}H_{28}N_6O_4 \cdot 2C_7H_8O_3S \cdot 1/2H_2O$	52.30 (52.22	5.98 6.46	11.09 10.53)	0.24
H-Tyr(Et)-Har-OMe·2HCl	75	197—199	+19.0	$C_{19}H_{30}N_5O_4 \cdot 2HCl \cdot 1/2H_2O$	48.09 (48.33	7.01 7.28	14.76 14.74)	0.31
H-Trp(CHO)-Har-OMe·2HCl	70	170—174 (dec.)	+ 5.0	$C_{20}H_{28}N_6O_4 \cdot 2HCl$	49.07 (48.60	6.18 6.14	17.17 17.24)	0.27
H-Tyr(*n*-Pr)-Har-OMe·2HCl	76	192—195 (dec.)	+12.0	$C_{20}H_{33}N_5O_4 \cdot 2HCl \cdot 1/2H_2O$	49.46 (49.25	7.41 7.40	14.30 14.14)	0.32
H-Tyr(iso-Pr)-Har-OMe·2HCl	65	180—184 (dec.)	+18.0	$C_{20}H_{33}N_5O_4 \cdot 2HCl \cdot 1/2H_2O$	49.07 (49.36	7.41 7.69	14.31 14.07)	0.32
H-Tyr(*n*-Pr)-Har-OMe·2HCl	67	200—203 (dec.)	+20.0	$C_{21}H_{35}N_5O_4 \cdot 2HCl$	51.00 (50.96	7.54 7.64	14.16 13.86)	0.35
Z-Tyr(*tert*-Bu)-Har-OMe	78	110—114	+26.0	$C_{29}H_{41}N_5O_6 \cdot H_2O$	60.71 (59.85	7.56 7.16	12.21 11.78)	0.77

a) Optical rotations were measured in MeOH ($c=1$) at 20—23°C unless otherwise noted.
b) Found values in parentheses.
c) See Experimental.
d) The compound was purified by column chromatography on Dowex 1×2 (acetate form) using H_2O as an eluent and on Sephadex LH-20 using 50% EtOH as an eluent.
e) See ref. 3.

reported by Klausner *et al.*[13] gave Boc–Gph–OEt, which, after removal of the Boc group with 4 N HCl–dioxane, was condensed with Boc–Tyr(Bzl)–OH to give a dipeptide intermediate, Boc–Tyr(Bzl)–Gph–OEt. In order to synthesize *O*-alkylated Tyr analogs, **27** and **29**, H–Tyr(*n*-Pr)–OH and H–Tyr(*n*-Bu)–OH[14] were prepared by *O*-alkylation of H–Tyr–OH with the appropriate alkyl halide using NaOH in H_2O/DMSO solution according to a method reported by Solar and Schumaker.[14] *tert*-Butoxycarbonylation of these *O*-alkyl Tyr derivatives and subsequent condensation with H–Har(HCl)–OMe gave dipeptide intermediates, Boc–Tyr(*n*-Pr)–Har–OMe and Boc–Tyr(*n*-Bu)–Har–OMe, respectively. For the synthesis of **28**, Boc–Tyr(iso-Pr)–OH,[15] prepared by the method of Kolodziejczyk and Manning,[15] was condensed with H–Har(HCl)–OMe to give Boc–Tyr(iso-Pr)–Har–OMe. For the synthesis of **30**, Z–Tyr(*tert*-Bu)–Har–OMe, prepared from Z–Tyr(*tert*-Bu)–OH[16] and H–Har(HCl)–OMe, was adopted as the dipeptide intermediate and the Z group of the intermediate was removed by catalytic hydrogenation for the cyclization. All the dipeptide intermediates and their physical constants are listed in Table II.

After cyclization of the dipeptide ester by the method mentioned above, the crude product was purified by ion-exchange column chromatography on CM-Sepharose and/or partition column chromatography on Sephadex G-25.[17] The side chain protecting group (Z or Bzl) was removed by catalytic hydrogenation prior to purification.

In the case of the synthesis of **17**, cyclo(–Tyr–Dab–) (**20**) was guanidylated by the method of Habeeb[18] in 0.1 M $NaHCO_3$–Na_2CO_3 buffer at pH 9.5 and the desired product was purified by Sephadex G-10 gel filtration, partition column chromatography on Sephadex G-25 and ion-exchange column chromatography on CM-Sepharose. Analog **23** was also prepared from the corresponding diketopiperazine, cyclo[–Tyr–Arg(HCl)–],[1] by reaction with 1,2-cyclohexanedione[19] in 0.1 M borate buffer at pH 9.0, followed by Sephadex G-10 gel filtration and ion-exchange column chromatography on CM-cellulose. The purity of all diketopiperazines thus obtained was checked by TLC using two different solvent systems and by elemental analysis. Table III shows the yields and physical constants of all diketopiperazines.

Table III. Yields and Physical Constants of Diketopiperazines

Analog	Yield (%)	mp (°C)	$[\alpha]_D$[a]	Formula	Analysis (%)[b] Calcd (Found) C	H	N	*Rf*[c] (A)	(B)
1	74	179—184	− 40.9 ($c=0.2$)	$C_{15}H_{27}N_5O_2 \cdot 3/2CH_3COOH \cdot 3/2H_2O$	50.68 (50.50)	8.51 8.60	16.42 16.27)	0.51	0.86
2	88	170—177 (dec.)	− 29.0	$C_{12}H_{23}N_5O_2 \cdot 3/2CH_3COOH \cdot 3/2H_2O$	47.79 (47.91)	8.56 8.83	20.52 20.78)	0.39	0.83
3	77	176—178	− 56.0	$C_{11}H_{21}N_5O_2 \cdot 5/4CH_3COOH$	49.07 (48.80)	7.93 8.08	21.20 21.29)	0.38	0.83
4	32	107—110	− 87.0	$C_9H_{17}N_5O_3 \cdot 3/2CH_3COOH \cdot H_2O$	41.10 (40.97)	7.17 7.39	19.93 20.26)	0.14	0.50
5	30	114—120	− 18.2	$C_9H_{17}N_5O_2 \cdot 3/2CH_3COOH \cdot H_2O$	44.16 (44.10)	7.43 7.82	21.45 21.46)	0.24	0.56
6[d]	70	183—186 (dec.)	+ 13.7 ($c=0.7$, 80% MeOH)	$C_{15}H_{21}N_5O_2 \cdot HCl \cdot 3/2H_2O$	49.10 (49.43)	6.87 6.41	19.09 18.97)	0.41	0.69
7	59	129—134	− 13.0	$C_{17}H_{22}N_6O_2 \cdot CH_3COOH \cdot 3/2H_2O$	53.13 (53.52)	6.81 6.74	19.59 19.26)	0.39	0.68
8	47	170—175	− 62.5 ($c=0.5$)	$C_{17}H_{22}N_6O_3 \cdot CH_3COOH \cdot 2H_2O$	50.21 (50.25)	6.65 6.68	18.49 18.77)	0.32	0.54
9	84	131—134	+ 15.0	$C_{16}H_{23}N_5O_3 \cdot 3/2CH_3COOH \cdot 5/4H_2O$	51.17 (51.11)	7.12 7.28	15.70 15.68)	0.37	0.68
10	48	136—138	+ 40.0 ($c=0.5$)	$C_{17}H_{25}N_5O_3 \cdot CH_3COOH \cdot 1/2H_2O$	54.79 (54.74)	7.28 7.42	16.80 16.68)	0.38	0.73
11	69	212—216	+ 33.0 ($c=0.7$)	$C_{15}H_{19}N_5O_3Br_2 \cdot CH_3COOH \cdot H_2O$	36.77 (36.73)	4.54 4.45	12.61 12.50)	0.46	0.68

Analog	Yield (%)	mp (°C)	$[\alpha]_D$[a]	Formula	Analysis(%)[b] Calcd (Found) C	H	N	Rf[c] (A)	(B)
12	66	121—123	− 30.0 (c=0.8)	$C_{18}H_{22}N_6O_3\cdot 3/2CH_3COOH$	52.22 (52.45	6.20 6.67	17.82 17.68)	0.31	0.65
13	60	117—121	+ 51.0	$C_{16}H_{22}N_5O_3\cdot CH_3COOH\cdot 5/4H_2O$	51.97 (51.94	7.14 7.39	16.84 16.44)	0.33	0.58
14	88	88— 91	− 25.0 (c=0.4)	$C_{18}H_{26}N_5O_3\cdot 3/2CH_3COOH\cdot H_2O$	53.83 (54.30	7.32 7.57	14.95 14.55)	0.39	0.76
15	78	182—187	+ 52.6 (c=0.4)	$C_{16}H_{23}N_5O_3\cdot CH_3COOH\cdot H_2O$	52.54 (52.02	7.10 6.82	17.02 16.62)	0.35	0.73
16	65	178—181	−116.4 (c=0.7)	$C_{18}H_{21}N_5O_3\cdot 3/2CH_3COOH\cdot 1/2H_2O$	55.49 (55.45	6.21 6.20	15.41 15.35)	0.48	0.76
17	51	279—286 (dec.)	+ 26.7 (c=0.5)	$C_{14}H_{19}N_5O_3\cdot CH_3COOH\cdot 2H_2O$	47.87 (47.88	6.78 6.21	17.45 17.45)	0.36	0.70
18	77	205—210 (dec.)	+ 29.0	$C_{15}H_{21}N_3O_3\cdot CH_3COOH\cdot 3/2H_2O$	53.95 (53.78	7.47 7.12	11.10 11.24)	0.28	0.72
19	72	215—220 (dec.)	+ 62.5 (c=0.4)	$C_{14}H_{16}N_2O_3\cdot CH_3COOH\cdot 2H_2O$	51.46 (51.68	7.29 6.86	11.25 11.60)	0.27	0.72
20	89	203—205	+ 66.0 (c=0.3)	$C_{13}H_{17}N_3O_3\cdot CH_3COOH$	52.77 (52.94	6.79 6.75	12.31 12.31)	0.28	0.71
21[e]	58	198—202 (dec.)	− 49.0 (AcOH)	$C_{15}H_{16}N_4O_3\cdot 1/2CH_3COOH\cdot 1/2H_2O$	56.62 (56.34	5.64 5.62	16.51 16.22)	0.38	0.76
22[f]	89	151	−148.0	$C_{14}H_{16}N_2O_3\cdot H_2O$	64.60 (64.50	6.20 6.28	10.76 10.70)	0.83	0.89
23	64	160—170 (dec.)	+ 75.8 (c=0.3, 1% AcOH)	$C_{21}H_{30}N_5O_5\cdot CH_3COOH$	56.08 (55.73	6.96 6.75	14.22 13.72)	0.45	0.78
24	20	166—168	+ 12.0	$C_{18}H_{24}N_6O_3\cdot CH_3COOH\cdot H_2O$	53.32 (53.72	6.71 6.82	18.65 18.36)	0.34	0.68
25	44	240—245 (dec.)	+ 18.0	$C_{18}H_{27}N_5O_3\cdot CH_3COOH\cdot H_2O$	54.65 (54.64	7.57 7.76	15.94 15.85)	0.38	0.75
26	78	129—132	− 8.0	$C_{19}H_{24}N_6O_3\cdot CH_3COOH\cdot H_2O$	54.53 (54.51	6.54 6.56	18.17 18.16)	0.35	0.71
27	53	161—163	+ 23.4	$C_{19}H_{29}N_5O_3\cdot CH_3COOH\cdot 3/2H_2O$	54.53 (54.66	7.85 8.01	15.14 15.08)	0.39	0.75
28	70	158—160	+ 26.0	$C_{19}H_{29}N_5O_3\cdot CH_3COOH\cdot 2H_2O$	53.49 (53.16	7.91 7.60	14.85 14.93)	0.38	0.74
29	40	155—157	+ 25.0	$C_{20}H_{31}N_5O_3\cdot CH_3COOH\cdot 3/2H_2O$	55.44 (55.27	8.04 7.71	14.70 14.60)	0.42	0.73
30	38	129—132	+ 20.5 (c=0.5)	$C_{20}H_{31}N_5O_3\cdot CH_3COOH\cdot 3/2H_2O$	55.44 (55.76	8.04 8.35	14.70 14.46)	0.40	0.75

a) Optical rotations were measured in H_2O (c=1) at 20—23°C unless otherwise noted.
b) Found values in parentheses.
c) See Experimental.
d) The hydrochloride was purified by recrystallization from a small amount of H_2O.
e) Lit.[K.D. Kopple, R.R. Jarabak, and P.L. Bhatia, *Biochemistry*, **2**, 958 (1963).], mp 272—276°C (dec.), $[\alpha]_D^{25}$ −52.3° ± 0.3° (c=12.9, AcOH).
f) Lit.[P.E. Young, V. Madison, and E.R. Blout, *J. Am. Chem. Soc.*, **98**, 5365 (1976).], mp 152—154°C.

The analgesic effects of synthetic diketopiperazines after intracerebral administration to mice were evaluated in essentially the same manner as described previously.[1,20] The ED_{50} values of all analogs are listed in Table I and compared with that of cyclo(-Tyr-Arg-), Met-enkephalin or morphine on a molar basis.

Diketopiperazines in which Tyr was replaced by an aliphatic amino acid, **1**, **2**, **3**, **4** and **5**, showed 50—90% of the activity of cyclo(-Tyr-Arg-), while diketopiperazines in which Tyr was replaced by another aromatic amino acid (Phe or Trp), **6** and **7**, showed 70—80% of the activity of cyclo(-Tyr-Arg-). These observations indicate that the aromatic nucleus of the Tyr moiety is not essential for the analgesic activity. Diketopiperazines in which Tyr was replaced by an aromatic amino acid having a substituent group(s) on the aromatic nucleus, **8**, **9**, **10**, **11** and **12**, showed remarkably increased activity, 1.8 to 10 times that of cyclo(-Tyr-

Arg–). N^{α}-Alkylated Tyr analogs, **13** and **14**, were 1.5 times as potent as cyclo(–Tyr–Arg–). The activity of diketopiperazines in which Arg was replaced by another basic amino acid decreased in the order **15**, **16**, **17**, **18**, **19**, **21** and **20**. Analog **22** was inactive. Partial masking of the basicity of the guanidino group in Arg with a DHCH group (**23**) resulted in slightly less activity. Observations on this series of analogs indicate that the basicity of the guanidino group is a dominant factor for the activity, and most long side chains in Har and Gph among the diketopiperazines tested contributed to an increased activity.

In the third series of analogs, the *O*-ethylated Tyr analog, **25**, showed the most potent activity among analogs tested in this study, that is, it was 17 times more active than cyclo(–Tyr–Arg–). This activity is nearly comparable to that of morphine on a molar basis. Further extension of the alkyl chain (**27** and **29**) or introduction of a bulky alkyl chain (**28** and **30**) in *O*-alkylated Tyr analogs tended to decrease the activity.

The analgesic effects of these diketopiperazines reached a maximum within 5 min and there were no abnormal behavioral changes such as sedation or convulsion.

Experimental

All melting points are uncorrected. TLC was performed on silica gel plates (Kieselgel GF_{254}, Merck) with the following solvent systems: *Rf*(A), 1-BuOH–AcOH–H_2O (4: 1: 5, upper phase); *Rf*(B), 1-BuOH–pyridine–AcOH–H_2O (15: 10: 3: 12). The Boc group of dipeptide intermediates in Table II was removed by 4 N HCl–dioxane treatment before TLC. Optical rotations were determined with an Atago Polax. Infrared (IR) spectra were recorded with a Shimadzu IR-430 spectrophotometer. Paper electrophoresis was performed on Toyo Roshi No. 51 paper in pH 6.45 pyridinium acetate buffer at 500 V for 60 min.

Boc-Tyr(Et)-OH——H–Tyr(Et)–OH was prepared from N^{α}-acetyl-Tyr–OH with $(C_2H_5O)_2SO_4$ by a method similar to that described for H–Tyr(Me)–OH by Siedel *et al.*,[6] and recrystallized from H_2O. Yield 74%; mp 224—228°C (dec.); $[\alpha]_D^{20}$ −14.4° (*c*=0.8, 1 N HCl); *Rf*(A) 0.47, *Rf*(B) 0.68; *Anal.* Calcd for $C_{11}H_{15}NO_3$: C, 63.14; H, 7.23; N, 6.69. Found: C, 63.32; H, 7.31; N, 6.48. The title compound was obtained from the above product by *tert*-butoxycarbonylation with Boc–ON[7] in a usual manner; yield 60%; mp 81—84°C; $[\alpha]_D^{20}$ +32.6° (*c*=1.0, MeOH) (Lit.[15] mp 90—92°C, $[\alpha]_D^{20}$ +39.8° (*c*=1, EtOH)); *Anal.* Calcd for $C_{16}H_{23}NO_5$: C, 62.12; H, 7.49; N, 4.53. Found: C, 62.10; H, 7.70; N, 4.46.

Tfa-Tyr(Bzl)-OBzl——*S*-Ethyl thioltrifluoroacetate[21] (2.5 ml) was added to a solution of H–Tyr(Bzl)–OBzl (3.4 g), derived from the tosylate,[22] in EtOAc (50 ml), and the mixture was stirred at room temperature overnight, then the solution was evaporated to dryness *in vacuo*. The resulting product was dissolved in abs. ether (15 ml) and stored in the cold. The precipitate thereby formed was filtered off and the filtrate was evaporated to dryness. The product was recrystallized from abs. ether–pet. ether to give colorless needles, wt. 3.66 g (85%); mp 83—84°C; $[\alpha]_D^{20}$ −15.0° (*c*=1.0, MeOH); *Anal.* Calcd for $C_{25}H_{21}F_3NO_4$: C, 65.64; H, 4.85; N, 3.06. Found: C, 65.37; H, 4.89; N, 3.33.

Boc-AlTyr-OH——Powdered KOH (460 mg) was added to a solution of Tfa–Tyr(Bzl)–OBzl (750 mg) and allyl bromide (0.68 ml) in acetone (5 ml), and the mixture was warmed nearly to reflux for 40 min, then AcOH (0.5 ml) was added and the whole was evaporated to dryness. The residue was extracted with EtOAc (×2) and the extract was washed well with H_2O and concentrated *in vacuo*. The resulting oily residue was dissolved in 6 N HCl (10 ml) containing anisole (0.5 ml) and the solution was heated at 110°C for 22 h in an open flask. The solution, after being washed with ether (×3), was evaporated to dryness. The product thus obtained was dissolved in a small amount of 1-BuOH–AcOH–H_2O (4: 1: 5, upper phase) and applied to a column (2.8×45 cm) of Sephadex G-25, which was pre-equilibrated and eluted with the same solvent. Fractions of 6.2 ml each were collected and tubes No. 40—60 were pooled and evaporated to dryness. The product was dissolved in 50% aqueous EtOH (10 ml) and the pH was adjusted to 6 with 1 N NH_4OH. Storage of the solution in the cold gave H–AlTyr–OH as colorless fine needles, wt. 200 mg (45%); mp 242—244°C (dec.); $[\alpha]_D^{20}$ +62.3° (*c*=0.9, 1 N HCl). *Rf*(A) 0.47. IR ν_{max}^{Nujol} cm^{-1}: 1645, 990, 940. *Anal.* Calcd for $C_{12}H_{15}NO_3$: C, 65.14; H, 6.83; N, 6.33. Found: C, 65.17; H, 7.07; N, 6.23. *tert*-Butoxycarbonylation of the product with Boc–ON gave the title compound as an oil, which was converted to the dicyclohexylamine salt in a usual manner; Yield 70%; mp 138—140°C; $[\alpha]_D^{20}$ −29.1° (*c*=0.9, MeOH). IR ν_{max}^{Nujol} cm^{-1}: 1665, 975, 920; *Anal.* Calcd for $C_{17}H_{23}NO_5 \cdot C_{12}H_{23}N$: C, 69.29; H, 9.22; N, 5.57. Found: C, 69.34; H, 9.41; N, 5.56.

Boc-Phe(NO_2)-OEt——Powdered KF (871 mg) was added to a solution of C_2H_5Br (0.38 ml) in DMF (10 ml), followed by addition of Boc–Phe(NO_2)–OH[11] (1.55 g). The resulting mixture was stirred at room temperature overnight and, after dilution with H_2O (60 ml), was extracted with EtOAc (×2). The extract was washed well with 1 N $NaHCO_3$ and H_2O, dried over $MgSO_4$ and evaporated to dryness. The product was recrystallized from EtOAc–*n*-hexane to give colorless needles, wt. 1.4 g (83%); mp 61°C; $[\alpha]_D^{20}$ −20.0°

(c=1.0, MeOH); de-Boc derivative, Rf(A) 0.58, Rf(B) 0.85. *Anal.* Calcd for $C_{16}H_{22}N_2O_6$: C, 56.79; H, 6.55; N, 8.28. Found: C, 56.82; H, 6.69; N, 8.30.

Boc-Gph-OEt——Boc-Phe(NO_2)-OEt (1.01 g) was dissolved in MeOH (27 ml) and AcOH (0.2 ml) and hydrogenated in the presence of 10% Pd/C (170 mg) under ice-cooling for 4 h. After removal of the catalyst through celite, the solution was evaporated to dryness to give an oil, which was dried over KOH pellets *in vacuo*. The product was dissolved in THF (7 ml) containing diisopropylamine (0.8 ml), followed by addition of 1-amidino-3,5-dimethylpyrazole nitrate (905 mg) and the solution was refluxed for 16 h. After removal of the solvent by evaporation, the resulting oil was dissolved in MeOH (15 ml) containing AcOH (0.8 ml) and stored in the cold. The precipitate thereby formed was filtered off and the filtrate was concentrated *in vacuo* to give an oil, which was crystallized under abs. ether. Recrystallization from EtOH–EtOAc gave colorless fine needles, wt. 575 mg (55%); mp 131—133°C; Rf(A) 0.66, Rf(B) 0.84, single spot positive to Sakaguchi and negative to ninhydrin reagent; $[\alpha]_D^{21}$ −21.1° (c=0.8, MeOH). *Anal.* Calcd for $C_{17}H_{26}N_4O_4 \cdot H_2O$: C, 55.42; H, 7.66; N, 15.21. Found: C, 55.26; H, 7.53; N, 15.54.

H-Har(HCl)-OMe·HCl——This compound was prepared by esterification of H-Har-OH with $SOCl_2$ in MeOH,[23] and reprecipitated from MeOH–abs. ether. Yield 90%, mp 118—120°C; $[\alpha]_D^{21}$ +34.0° (c=1.0, MeOH); Rf(A) 0.13, Rf(B) 0.48. *Anal.* Calcd for $C_8H_{18}N_4O_2 \cdot 2HCl$: C, 35.16; H, 6.64; N, 20.51. Found: C, 34.64; H, 5.57; N, 20.20.

H-Tyr(*n*-Pr)-OH——This compound was prepared by *O*-propylation of H-Tyr-OH with *n*-propyl bromide in the presence of 10% NaOH in aqueous DMSO by the method of Solar and Schumaker,[14] and recrystallized from H_2O. Yield 48%; mp 228—230°C (dec.); $[\alpha]_D^{22}$ −39.0° (c=1.3, 1 N HCl); *Anal.* Calcd for $C_{12}H_{17}NO_3 \cdot 1/2H_2O$: C, 62.05; H, 7.81; N, 6.03. Found: C, 61.86; H, 7.42; N, 6.02.

Boc-Tyr(*n*-Bu)-OH——This compound was prepared by *tert*-butoxycarbonylation of H-Tyr(*n*-Bu)-OH[14] with Boc-ON[7] in a usual manner as an oil, which was converted to the dicyclohexylamine salt in a usual manner. Yield 90%; mp 113—115°C; $[\alpha]_D^{22}$ +37.2° (c=1.0, MeOH). *Anal.* Calcd for $C_{18}H_{27}NO_5 \cdot C_{12}H_{23}N$: C, 69.46; H, 9.72; N, 5.40. Found: C, 68.92; H, 9.90; N, 5.28.

Preparation of Dipeptide Intermediates——In general, H-Arg(HCl)-OMe or H-Har(HCl)-OMe was coupled with the corresponding Boc-amino acid by the DCC–HOBt or WSCI–HONB method in a usual manner. After removal of DC-urea by filtration, the filtrate was diluted with 5 volumes of H_2O and extracted with H_2O-saturated 1-BuOH (×2). The extract was washed with 1-BuOH-saturated 1 N AcOH, 1-BuOH-saturated 1 N NH_4OH and 1-BuOH-saturated H_2O, then evaporated to dryness to give an oily residue, which was dissolved in fresh 1-BuOH and again evaporated to dryness *in vacuo*. The resulting residue was triturated with abs. ether to give a solid mass, which was collected and dried over P_2O_5 *in vacuo*. When the product was an oil, the residue was treated with 4 N HCl–dioxane to give the dipeptide ester dihydrochloride, which, in many cases, was recrystallized from EtOH or 2-propanol (see Table II).

In the preparations of Boc-Tyr(Bzl)-Lys(Z)-OMe, Boc-Orn(Z)-Tyr-OMe, Boc-Dab(Z)-Tyr-OMe and Boc-Tyr-Pro-OBzl, the desired product was extracted with EtOAc (×2). The extract was washed well with 1 N $NaHCO_3$, 1 N citric acid, H_2O, and, after being dried over $MgSO_4$, was concentrated *in vacuo*. The resulting product was recrystallized from EtOAc–pet. ether.

Preparation of Diketopiperazines——The Boc group of dipeptide intermediates was removed by 4 N HCl–dioxane treatment except for Trp or 5HTP-containing dipeptide intermediates, which were treated with 2 M *p*-toluenesulfonic acid in dioxane containing anisole (5%),[24] and cyclization of the resulting dipeptide esters was carried out by the acetic acid-catalyzed method.[3] In general, after evaporation of the reaction solvent *in vacuo*, the residue was dissolved in a small amount of H_2O and passed through a column of Dowex 1×2 resin (acetate form), which was eluted with H_2O. The eluates containing the desired product were pooled and lyophilized. In cases where the diketopiperazine had a protecting group (Bzl or Z) the product was hydrogenated in the presence of 10% Pd/C in MeOH–H_2O (1: 1) in a usual manner prior to the Dowex 1×2 resin treatment. The product was applied to a column of CM-Sepharose, which was eluted with a gradient formed with 0.1 M pyridine–acetate buffer (pH 5.20, 300 ml) through a mixing chamber containing H_2O (300 ml). The eluate was monitored by a suitable method such as ultraviolet (UV) absorption measurement or detection with Cl-*o*-tolidine reagent. Main fractions containing the desired product were pooled and lyophilized. When a contaminant(s) was detected on TLC, the product was further purified by partition column chromatography on Sephadex G-25 as described for the preparation of Boc-AlTyr-OH.

Cyclo(-Tyr-Gbu-) (17)——A solution of cyclo(-Tyr-Dab-) (60 mg) in 0.1 M $NaHCO_3$–Na_2CO_3 buffer (pH 9.5, 10 ml) was combined with a cooled solution of 1-amidino-3,5-dimethylpyrazole nitrate (1 g) in 2 N NaOH (5 ml) and the solution was stirred at 5°C for 60 h. The pH was maintained at 9.5—9.8 with 1 N NaOH throughout the reaction. A few drops of 5 N HCl were added, then the solution was concentrated to a small volume and applied to a column (2.5×80 cm) of Sephadex G-10, which was eluted with 1% AcOH. Fractions of 6.5 ml each were collected and tubes No. 55—65 were pooled and lyophilized. The product was subjected to partition column chromatography on Sephadex G-25 as described for the preparation of Boc-AlTyr-OH. Fractions of 5.6 ml each were collected and tubes No. 65—96 were pooled and lyophilized. The product was dissolved in H_2O (5 ml) and treated with Dowex 1×2 (acetate form) resin (about 2 g by wet weight) for 30 min. After removal of the resin by filtration, the filtrate was concentrated to a small volume and applied to a column (2×10 cm) of CM-Sepharose, which was eluted with a linear gradient in the same manner as

described for the preparation of diketopiperazines. Fractions of 5.5 ml each were collected and tubes No. 46—52 were pooled, evaporated to dryness and lyophilized from H_2O to give colorless fluffy material, wt. 36 mg.

Cyclo[-Tyr-Arg(DHCH)-] (23)——A mixture of cyclo[-Tyr-Arg(HCl)-][1] (56 mg) in 0.1 M borate buffer (pH 9.0, 5 ml) and 1,2-cyclohexanedione (20 mg) was stirred under N_2 gas at room temperature for 50 h, then a few drops of 5 N HCl were added. The resulting clear solution was evaporated to dryness and the residue was applied to a column (2.5 × 95 cm) of Sephadex G-10, which was eluted with 1 N AcOH. Fractions of 5.5 ml each were collected and tubes No. 70—82 were pooled and lyophilized. The product, after treatment with Dowex 1 × 2 (acetate form) resin as described above, was applied to a column (2 × 13 cm) of CM-cellulose. The column was eluted with a linear gradient formed from 0.1 M pyridine-acetate buffer (pH 5.00, 300 ml) through a mixing chamber containing H_2O (300 ml). Fractions of 6.3 ml each were collected and tubes No. 25—39 were pooled, evaporated to dryness and lyophilized from H_2O to give a colorless fluffy material, wt. 47 mg; electrophoretic mobility: $E_{C.(-Tyr-Arg-)}$ 0.76. This compound decomposed slowly in the solid state on storage at room temperature.

Analgesic Activity Assay——Analgesic activity was assessed in adult male ddY mice (20—24 g) using the tail pressure test.[25] The test compounds were dissolved in Ringer's solution and intracerebrally injected into unanesthetized mice (20 μl/mouse) according to the modified method of Brittain and Handley.[26] The base of the tail was pressed and only mice which showed biting or struggling behavior in the pressure range of 40—60 mmHg were used. Each assay was done with 10 mice and the analgesic effect was determined to be positive when the tail pressure threshold was increased to over 60—80 mmHg by injection of the test compound. Tail pressure thresholds were determined at 5, 15, 30, 45 and 60 min, except for Met-enkephalin, for which it was determined at 2, 5 and 10 min, following injection. The ED_{50} value of each test compound, estimated according to the method of Litchfield and Wilcoxon,[27] is shown in Table I.

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References and Notes

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