from virus-infected HeLa cells that were deficient in the host thymidine kinase ($TK^-$), and the cellular enzyme was obtained from normal $TK^+$ HeLa cells. The standard TK assay, outlined in detail in ref 1, measured radioactivity retained on DEAE paper disks after incubation of [$^3$H]TdR with enzyme and ATP as phosphate donor for 30 min at 37 °C. The HSV1, HSV2, and cellular TKs were assayed in the presence of TdR at 1, 2, and 3 $\mu$M, respectively. Inhibitors were dissolved in dimethyl sulfoxide and diluted into assay mixtures; control assays contained an equal amount of dimethyl sulfoxide.

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# Synthesis and Structure–Activity Relationships of Dynorphin A-(1–8) Amide Analogues

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In order to study the structure–activity relationships of dynorphin A-(1–8) amide [Dyn(1–8)-$NH_2$], 20 analogues were synthesized by the solution method. Their biological activities were determined in the three bioassays [guinea pig ileum (GPI), mouse vas deferens (MVD), and rabbit vas deferens (RVD)] and in the mouse tail-pinch test after intravenous administration. Some analogues that showed interesting activity in the bioassays and/or in the analgesic tests were further characterized in $\mu$-, $\delta$-, and $\kappa$-representative binding assays. The obtained data indicate that modification of the enkephalin segment to give metabolically stable analogues with high affinity and selectivity for the $\kappa$ receptor is strictly limited and that introduction of MeArg in position 7 protects the $Arg^6$–$Arg^7$ bond from enzymatic degradation without potency drop and change of opioid receptor selectivity. [$MeTyr^1$,$MeArg^7$,D-$Leu^8$]Dyn(1–8)-NHEt (18) [$IC_{50}$ (nM) = 0.3 (GPI), 7.4 (MVD), and 2.6 (RVD); tail pinch $ED_{50}$ (mg/kg) = 0.75] showed opioid activity similar to that of dynorphin A in the three bioassays and relatively high $\kappa$-receptor selectivity in the binding assays and produced a 2.5-fold more potent analgesic effect than morphine. [D-$Cys^2$-$Cys^5$,$MeArg^7$,D-$Leu^8$]Dyn(1–8)-NHEt (**20**) showed a 40–60-fold more potent opioid activity than 18 in the three bioassays and produced a 3.4-fold more potent analgesic effect than 18. In the binding assays, however, **20** showed higher affinity for $\mu$ and $\delta$ receptors than for the $\kappa$ receptor.

Dynorphin A (Dyn) isolated either from porcine pituitary[1,2] or from porcine duodenum[3] is a 17 amino acid opioid peptide containing the sequence of [Leu]enkephalin at its N-terminal. In the guinea pig ileum longitudinal muscle preparation both Dyn and its fragment, Dyn(1–13), are about 700-fold more potent than [Leu]enkephalin and are relatively insensitive to naloxone inhibition.[1,2] It has been postulated that Dyn is the endogenous ligand for the $\kappa$ opioid receptor.[4,5]

Recently, we demonstrated that Dyn(1–8) still shows opioid activity similar to that of Dyn in the presence of peptidase inhibitors and that a metabolically stable analogue of Dyn(1–8), [$MeTyr^1$,$MeArg^7$,D-$Leu^8$]Dyn(1–8)-NHEt (18), not only retains opioid receptor selectivity similar to that of Dyn but also produces a more potent analgesic effect than morphine even when administered subcutaneously into mice.[6,7] In the present study we have focussed our attention on the synthesis and study of structure–activity relationships of the Dyn(1–8)-$NH_2$ analogues. Although there have been several reports on modification and structure–activity relationships of Dyn fragments,[8-18,26] no previous systematic study to find

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**Table I.** Analytical Data of Dyn(1–8)-$NH_2$ Analogues

| | compound | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| no. | Tyr | Gly | Gly | Phe | Leu | Arg | Arg | Ile | $[\alpha]^{20}_D$[a] | $R_f$[b] |
| 1 | Me | | | | | | | D-Leu-$NH_2$ | −7.5 | 0.62 |
| 2 | H | D-Ala | | | | | | D-Leu-$NH_2$ | +15.0 | 0.69 |
| 3 | Me | D-Ala | | | | | | D-Leu-$NH_2$ | +13.9 | 0.68 |
| 4 | H | Sar | | | | | | D-Leu-$NH_2$ | −3.8 | 0.64 |
| 5 | Me | | Azgly | | | | | D-Leu-$NH_2$ | −0.3 | 0.59 |
| 6 | Me | | D-Ala | | | | | D-Leu-$NH_2$ | +8.6 | 0.56 |
| 7 | Me | | | Phe($p$-$NO_2$) | | | | D-Leu-$NH_2$ | +3.4 | 0.59 |
| 8 | Me | | | MePhe | | | | D-Leu-$NH_2$ | −17.2 | 0.68 |
| 9 | Me | | | D-Phe | | | | D-Leu-$NH_2$ | −12.0 | 0.67 |
| 10 | Me | | | | D-Leu | | | D-Leu-$NH_2$ | +31.5 | 0.71 |
| 11 | Me | | | | MeLeu | | | D-Leu-$NH_2$ | −11.5 | 0.61 |
| 12 | Me | | | | D-Ala | | | D-Leu-$NH_2$ | +26.4 | 0.53 |
| 13 | Me | | | | | homoArg | | D-Leu-$NH_2$ | −4.2 | 0.61 |
| 14 | Me | | | | | MeArg | | D-Leu-$NH_2$ | −19.7 | 0.59 |
| 15 | Me | | | | | | MeArg | D-Leu-$NH_2$ | −23.4 | 0.62 |
| 16 | Me | | | | | | | D-Leu-NHEt | +5.0 | 0.64 |
| 17 | Me | | | | | | | D-Leu-$NH(CH_2)_5CH_3$ | +1.0 | 0.68 |
| 18 | Me | | | | | | MeArg | D-Leu-NHEt | −21.8 | 0.70 |
| 19 | Me | | | Phe($p$-$NO_2$) | | | MeArg | D-Leu-NHEt | −16.6 | 0.69 |
| 20 | H | D-Cys | | | Cys | | MeArg | D-Leu-NHEt | −29.1 | 0.70 |

[a] $c$ 0.4 in 0.01 N HCl. [b] Thin-layer chromatography on silica gel. Solvent system: 1-butanol/pyridine/acetic acid/water (15:5:5:8).

metabolically stable analogues with potent opioid activity appears to have been done. As was described in a preceding paper,[7] a simple analogue of Dyn(1–8), [MeTyr[1],D-Leu[8]]Dyn(1–8)-$NH_2$ (1), which shows opioid receptor selectivity roughly similar to that of Dyn, produces an analgesic effect to some extent when administered intravenously into mice. The appearance of the analgesic activity, even when administered intravenously, is mainly due to the enhanced stability of the peptide against enzymatic degradation. In this study, therefore, 1 was chosen as a parent compound, N-terminal MeTyr and C-terminal D-Leu amide moieties were fixed in principle, and the other constituents were replaced in turn from position 2. A great number of enkephalin analogues have already been synthesized.[25] Some of them have been reported to produce potent analgesic effects in rodents. Accordingly, in the modification of the N-terminal region (1–5) of 1, the available structure–activity information on enkephalin analogues was taken into consideration.

## Synthesis

All of the analogues listed in Table I were synthesized by the classical solution method by combination of stepwise elongation with fragment condensation. The Gly residue in position 3 was chosen as the racemization-free fragment coupling point. Mixed-anhydride, HOSu-active ester and DCC/HOBt were employed for the coupling method. α-Amino groups were protected by Z or Boc groups. Side chain protecting groups were as follows: Tyr, $Cl_2Bzl$; Cys, MBzl; Arg, Tos. *N*-Methyl amino acid derivatives were synthesized according to the method of Cheung et al.[19] with the exception of Z-MeArg(Tos)-OH, which was prepared similarly to the method of Quitt et al.[20]

**Table II.** Analytical Data on Dyn(1–8)-$NH_2$ Analogues

| | amino acid analysis[a] | | | | | | | FAB-MS[b] |
|---|---|---|---|---|---|---|---|---|
| compd | Tyr | Gly | Phe | Leu | Arg | Ala | Cys | ($MH^+$) |
| 1 | | 1.95 | 1.00 | 1.96 | 1.97 | | | 994 |
| 2 | 0.95 | 1.07 | 1.00 | 1.97 | 1.99 | 1.03 | | 994 |
| 3 | | 1.01 | 1.00 | 1.96 | 1.98 | 1.00 | | 1008 |
| 4 | 0.89 | 1.00 | 1.00 | 1.98 | 2.00 | | | 994 |
| 5 | | 1.04 | 1.00 | 2.00 | 2.01 | | | 995 |
| 6 | | 1.01 | 1.00 | 2.00 | 2.01 | 1.01 | | 1008 |
| 7 | | 1.97 | | 2.00 | 2.01 | | | 1039 |
| 8 | | 1.92 | | 2.00 | 2.03 | | | 1008 |
| 9 | | 1.90 | 1.00 | 1.93 | 1.94 | | | 994 |
| 10 | | 1.93 | 1.00 | 1.95 | 1.97 | | | 994 |
| 11 | | 1.99 | 1.00 | 1.02 | 2.03 | | | 1008 |
| 12 | | 1.97 | 1.00 | 1.05 | 2.04 | 1.00 | | 952 |
| 13 | | 1.95 | 1.00 | 1.96 | 1.01 | | | 1008 |
| 14 | | 1.93 | 1.00 | 1.91 | 0.98 | | | 1008 |
| 15 | | 1.92 | 1.00 | 1.95 | 0.99 | | | 1008 |
| 16 | | 1.91 | 1.00 | 2.14 | 2.18 | | | 1022 |
| 17 | | 1.92 | 1.00 | 1.95 | 1.95 | | | 1078 |
| 18 | | 1.87 | 1.00 | 1.96 | 0.95 | | | 1036 |
| 19 | | 1.99 | | 2.00 | 0.99 | | | 1081 |
| 20 | 0.82 | 1.01 | 1.00 | 1.05 | 1.02 | | 1.89 | 1056 |

[a] The proportions of only the primary protein amino acids were calculated. [b] Found values are in agreement with calculated values.

All the protecting groups were removed with HF/anisole in the usual manner. Disulfide bond formation was performed by air oxidation at pH 8.0. Purification was achieved by high-performance liquid chromatography (HPLC) on Nucleosil 5C18 (2 × 25 cm) with $H_2O/CH_3CN$ containing 0.05–0.1% HCl. Homogeneity of the purified product was accessed by thin-layer chromatography (TLC) and analytical HPLC. Structure identification was achieved by amino acid analysis and fast atom bombardment mass spectroscopy (FAB) (Table II).

## Biological Activity and Binding Property

The analogues listed in Table I were tested in vitro in three isolated organ preparations [guinea pig ileum (GPI),[21] mouse vas deferens (MVD),[22] and rabbit vas deferens (RVD)[23]] and in vivo by mouse tail-pinch assay[24] after

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Table III. Biological Activities of Dyn(1–8)-$NH_2$ Analogues

| compd | GPI: $IC_{50}$,[a] nM | MVD: $IC_{50}$,[a] nM | RVD: $IC_{50}$,[a] nM | analgesia (iv):[b] $ED_{50}$, mg/kg |
|---|---|---|---|---|
| 1 | 1.4 ± 0.3 | 5.2 ± 0.2 | 14.3 ± 12.3 | 3.89 (2.08–7.27) |
| 2 | 6.9 ± 1.2 | 9.1 ± 0.8 | 45.5 ± 17.0 | 11.74 (9.61–14.34) |
| 3 | 13.9 ± 3.0 | 25.7 ± 3.3 | 85.5 ± 31.0 | >5.00 |
| 4 | 897 ± 156 | 1766 ± 253 | 738 ± 265 | >10.00 |
| 5 | 18.6 ± 6.3 | 62.4 ± 10.2 | 152 ± 61 | |
| 6 | 12.7 ± 1.1 | 210 ± 36 | 125 ± 45 | |
| 7 | 1.3 ± 0.3 | 3.8 ± 1.1 | 5.5 ± 1.8 | 1.23 (0.64–2.34) |
| 8 | 26.7 ± 1.8 | 121 ± 12 | 301 ± 60 | |
| 9 | >3000 | | >2000 | |
| 10 | 82.0 ± 13.4 | >1737 | 1183 ± 758 | >6.00 |
| 11 | 11.9 ± 2.5 | 25.3 ± 3.7 | 189 ± 73 | |
| 12 | 6000 ± 1400 | 9800 ± 2600 | 13000 ± 3000 | >20.00 |
| 13 | 2.6 ± 1.4 | 12.0 ± 2.1 | 24.0 ± 5.8 | >8.00 |
| 14 | 20.6 ± 4.7 | 114 ± 32 | 107 ± 38 | 4.54 (2.21–9.32) |
| 15 | 0.5 ± 0.1 | 4.8 ± 0.6 | 2.3 ± 0.53 | 2.04 (1.27–3.29) |
| 16 | 0.7 ± 0.14 | 4.6 ± 0.7 | 4.9 ± 1.3 | 3.34 (1.97–5.67) |
| 17 | 6.6 ± 1.7 | 22.3 ± 5.2 | 20.0 ± 8.2 | >7.00 |
| 18 | 0.3 ± 0.03 | 7.4 ± 2.5 | 2.6 ± 0.4 | 0.75 (0.25–2.26) |
| 19 | 0.5 ± 0.1 | 3.7 ± 0.5 | 9.1 ± 3.7 | 0.77 (0.35–1.71) |
| 20 | 0.007 ± 0.001 | 0.13 ± 0.01 | 0.06 ± 0.01 | 0.22 (0.11–0.52) |
| Dyn | 0.2 ± 0.03 | 3.0 ± 0.5 | 17.4 ± 6.7 | |
| morphine | 71.6 ± 5.0 | 736 ± 135 | inactive | 1.85 (0.84–4.09) |

[a] Results are the means ± SEM. [b] Analgesia of four doses of each compound was investigated. Each dose was tested on at least eight animals.

Table IV. Binding Assays of Dyn(1–8)-$NH_2$ Analogues[a]

| compd | [$^3$H]DAGO: $K_i^\mu$, nM | [$^3$H]DPDPE: $K_i^\delta$, nM | [$^3$H]U69,593: $K_i^\kappa$, nM |
|---|---|---|---|
| 1 | 2.81 ± 0.66 | 38.97 ± 5.89 | 1.45 ± 0.57 |
| 3 | 1.25 ± 0.23 | 29.63 ± 3.42 | 9.24 ± 2.23 |
| 7 | 1.01 ± 0.23 | 28.84 ± 3.86 | 0.77 ± 0.24 |
| 15 | 3.34 ± 0.57 | 53.86 ± 11.50 | 1.38 ± 0.33 |
| 18 | 4.51 ± 0.85 | 27.24 ± 10.66 | 1.91 ± 0.22 |
| 19 | 1.74 ± 0.36 | 18.04 ± 4.84 | 3.94 ± 1.82 |
| 20 | 0.85 ± 0.22 | 1.59 ± 0.19 | 3.02 ± 1.01 |
| Dyn | 7.16 ± 1.96 | 23.62 ± 5.93 | 1.16 ± 0.25 |
| morphine | 7.71 ± 1.13 | 629.51 ± 144.18 | 1549.49 ± 439.35 |

[a] Mean of five or six determinations ± SEM.

intravenous administration. In the GPI opioid effects are primarily mediated by $\mu$ receptors, whereas $\kappa$ receptors are also present in this tissue. The MVD assay is generally taken as being representative for $\delta$-receptor interactions, even though it also contains $\mu$ and $\kappa$ receptors. The RVD is considered to have only $\kappa$ receptors. Opioid receptor affinities of some analogues that showed interesting activity in the bioassays and/or in the analgesic tests were determined by displacement of selective radioligands from guinea pig brain membrane binding sites. [$^3$H]DAGO[29] was used as $\mu$ ligand, [$^3$H]DPDPE[30] was used as $\delta$ ligand, and [$^3$H]U69,593[31] was used as $\kappa$ ligand. The results are shown in Tables III and IV, in comparison with those of Dyn and morphine.

## Results and Discussion

Dyn has a [Leu]enkephalin segment at its N-terminal, whereas the receptor specificity of the former is quite different from that of the latter. Thus, it is of particular interest to examine whether the modification to increase opioid activity of enkephalin is applicable to Dyn(1–8)-$NH_2$ analogues without a drop in opioid activity and a change in receptor selectivity.

It has been reported that in the modification of enkephalin replacement of Gly$^2$ by D-Ala increases the opioid activity and analgesic effect of the peptide.[32,33] With respect to Dyn fragment analogues containing D-Ala$^2$, Paterson et al. demonstrated that the replacement of Gly by D-Ala in position 2 of Dyn(1–9) caused a substantial decrease in $\kappa$ binding whereas it increases $\mu$ and $\delta$ binding.[13] On the other hand, Walker et al. revealed that, although [D-Ala$^2$]Dyn(1–13)-$NH_2$ showed almost the same affinity for $\mu$,$\delta$, and $\kappa$ receptors, further substituted analogue, [D-Ala$^2$,(F$_5$)Phe$^4$]Dyn(1–13)-$NH_2$, showed $\kappa$-receptor selectivity comparable to that of Dyn(1–13).[16] Therefore, biological activity of [D-Ala$^2$,D-Leu$^8$]Dyn(1–8)-$NH_2$ (**2**) was first examined. As compared with [MeTyr$^1$,D-Leu$^8$]Dyn-(1–8)-$NH_2$ (**1**), **2** was 5-fold less potent on the GPI, 2-fold less potent on the MVD, and 3-fold less potent on the RVD (Table III). Replacement of Gly in position 2 of **1** by D-Ala to give [MeTyr$^1$,D-Ala$^2$,D-Leu$^8$]Dyn(1–8)-$NH_2$ (**3**) also caused a potency drop in the three bioassays. These bioassay data appear to indicate that **2** and **3** retain $\kappa$-receptor selectivity similar to that of **1** although drops in potency occurred. In the opioid receptor binding assays, however, **3** displayed 2-fold higher $\mu$-receptor affinity and 6-fold lower $\kappa$-receptor affinity as compared with **1**, indicating that **3** no longer has $\kappa$-receptor selectivity but $\mu$-receptor selectivity instead (Table IV). The reason for this apparent discrepancy between bioassay and binding assay data is unclear at present. Introduction of Sar in position 2 of [Leu]enkaphalin was reported to cause a drastic de-

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crease on the GPI and MVD.[25] Likewise, [Sar$^2$,D-Leu$^8$]-Dyn(1-8)-$NH_2$ (4) showed negligible activity in the three bioassays. Substitution of Azgly in position 3 of [Leu]-enkephalin methyl ester was demonstrated not to affect potency significantly on the GPI,[25] whereas [MeTyr$^1$,Azgly$^3$,D-Leu$^8$]Dyn(1-8)-$NH_2$ (5) showed less than one-tenth the potency of 1 in the three bioassays. Replacement of Gly in position 3 of 1 by D-Ala led to [MeTyr$^1$,D-Ala$^3$,D-Leu$^8$]Dyn(1-8)-$NH_2$ (6) with remarkably reduced potency in the three bioassays as in the case of D-Ala$^3$ substitution of [Leu]enkephalin.[25] [Phe(*p*-$NO_2$)$^4$,Leu$^5$]enkephalin was reported to be a potent analogue of [Leu]enkephalin.[26] On the other hand, introduction of Phe(*p*-$NO_2$) in position 4 of Dyn(1-13) was demonstrated to produce a 4-fold drop in potency on the GPI.[26] However, [MeTyr$^1$,Phe(*p*-$NO_2$)$^4$,D-Leu$^8$]Dyn(1-8)-$NH_2$ (7), as compared with 1, showed similar activity on the GPI and MVD and 2-fold more potent activity on the RVD. These bioassay data appear to indicate that the $\kappa$-receptor selectivity was retained or slightly increased. In the binding assays, however, 7 showed about 3-fold higher $\mu$-receptor affinity and 2-fold higher $\kappa$-receptor affinity as compared with 1, indicating that 7 has high affinity for $\mu$ receptor as well as for $\kappa$ receptor. Its analgesic activity was increased 3-fold, which is comparable to that of morphine: $ED_{50}$ = 1.23 mg/kg for 7 and 1.85 mg/kg for morphine. A potent enkephalin analogue, [D-Ala$^2$,MePhe$^4$,Met(O)-ol$^5$]enkephalin[27] has MePhe in position 4, whereas replacement of Phe of 1 by MePhe to give [MeTyr$^1$,MePhe$^4$,D-Leu$^8$]Dyn-(1-8)-$NH_2$ (8) caused about a 20-fold potency decrease in the three bioassays. Substitution of D-Phe in position 4 of 1 to give [MeTyr$^1$,D-Phe$^4$,D-Leu$^8$]Dyn(1-8)-$NH_2$ (9) led to a dramatic potency decrease in the three bioassays as in the case of D-Phe$^4$ substitution of [Met]enkephalin.[25] [D-Ala$^2$,D-Leu$^5$]enkephalin and [MeLeu$^5$]enkephalin amide were reported to be enkephalin analogues with increased activity,[25] whereas introduction of D-Leu and MeLeu in position 5 of 1 to give [MeTyr$^1$,D-Leu$^{5,8}$]Dyn(1-8)-$NH_2$ (10) and [MeTyr$^1$,MeLeu$^5$,D-Leu$^8$]Dyn(1-8)-$NH_2$ (11), respectively, caused a notable potency decrease in the three bioassays. [MeTyr$^1$,D-Ala$^5$,D-Leu$^8$]Dyn(1-8)-$NH_2$ (12), which has a less bulky D-amino acid (D-Ala) in position 5, showed negligible activity.

On the basis of these results, it is conceivable that modification that reduces opioid activity of enkephalin is also detrimental in the case of Dyn(1-8)-$NH_2$ and that only part of the modification to increase opioid activity of enkephalin (e.g. MeTyr$^1$) is successfully applicable to Dyn(1-8)-$NH_2$.

Our previous study indicated that the Arg-Arg bond of Dyn is very susceptible to proteolytic cleavage in the mouse serum and brain homogenate.[6,7] Therefore, in order to find more potent analogues of 1, this bond must be protected from enzymatic degradation without a significant conformational change in the peptide and steric interference at the receptor. Replacement of Arg in position 6 of 1 by homoArg to give [MeTyr$^1$,homoArg$^6$,D-Leu$^8$]Dyn(1-8)-$NH_2$ (13) produced about a 2-fold drop in the three bioassays, indicating that lengthening of the side chain of Arg$^6$ by only one methylene group leads to a structure less suitable for interactions with receptors or within the molecule.

Amide bond involving homoArg was reported to be resistant to trypsin attack,[28] whereas the analgesic effect of 13 was also decreased more than 2-fold. The reason is not clear why the introduction of the trypsin-resistant homoArg in position 6 of 1 did not contribute to an increase in the in vivo activity. Substitution of MeArg in position 6 of 1 to give [MeTyr$^1$,MeArg$^6$,D-Leu$^8$]Dyn(1-8)-$NH_2$ (14) induced a 7-20-fold potency decrease in the three bioassays, whereas this modification left the analgesic activity of the peptide essentially unchanged. The higher in vivo activity than was expected on the basis of its in vitro activity appears to be due to the greatly increased stability of this compound against enzymatic degradation because a previous study[7] indicated that the Leu-Arg bond of Dyn is another site cleaved by peptidases. Replacement of Arg in position 7 of 1 by MeArg to give [MeTyr$^1$,MeArg$^7$,D-Leu$^8$]Dyn(1-8)-$NH_2$ (15) increased the potency 3-fold on the GPI and 6-fold on the RVD and did not affect potency on the MVD. In the binding assays, 15 showed almost the same receptor selectivity as 1. In addition, this substitution produced a 2-fold potency increase in the analgesic test. It is noteworthy that the duration of the analgesic effect was also prolonged more than 2-fold (duration; more than 2 h). It is evident, therefore, that introduction of MeArg in position 7 protects the peptide from attack of trypsin-like enzymes without producing a major conformational change of the peptide and steric interference at the receptor. Because replacement of Arg in position 6 or 7 of Dyn(1-13) analogues by D-Arg had been previously found to reduce the opioid activity remarkably (unpublished data), this replacement was not performed in this study.

Ethylation of a C-terminal amide of 1 to give [MeTyr$^1$,D-Leu$^8$]Dyn(1-8)-NHEt (16) did not significantly affect potency on the MVD but increased potency about 2-fold on the GPI and RVD. Its analgesic activity was nearly equal to that of 1. *n*-Hexylation of the C-terminal amide of 1 resulted in [MeTyr$^1$,D-Leu$^8$]Dyn(1-8)-NH-$(CH_2)_5CH_3$ (17) with reduced potency in both the bioassays and the analgesic test. Possibly, introduction of the lipophilic *n*-hexyl group, which is quite different in physicochemical character from the guanidine group of Arg in position 9 of Dyn, in the C-terminal amide of 1 induces conformational change of the peptide or steric interference at the receptor to some extent.

These results indicate that the in vitro and/or in vivo activity of 1 was enhanced by the introduction of Phe(*p*-$NO_2$)$^4$ or MeArg$^7$ or ethylation of C-terminal amide. In order to find more potent analogues, these effective modifications were combined in the same molecule. [MeTyr$^1$,MeArg$^7$,D-Leu$^8$]Dyn(1-8)-NHEt (18) showed almost the same opioid activity and receptor selectivity as 15, whereas the former produced a 2.7-fold more potent analgesic effect than the latter. This increase of in vivo activity may be due to the increased ability to permeate into the spinal cord or the brain. In any case, it is noteworthy that 18 produced a 2-fold more potent analgesic effect than morphine: $ED_{50}$ = 0.75 mg/kg for 18 and 1.85 mg/kg for morphine. Against expectation, additional introduction of Phe(*p*-$NO_2$) in position 4 of 18 to give [MeTyr$^1$,Phe(*p*-$NO_2$)$^4$,MeArg$^7$,D-Leu$^8$]Dyn(1-8)-NHEt (19) caused a significant drop in both the $\kappa$-opioid activity and the $\kappa$-receptor selectivity.

With respect to the conformationally restricted Dyn analogue, Schiller et al. noted that a cyclic enkephalin analogue, [D-Cys$^2$-Cys$^5$]enkephalinamide, was 163-fold more potent than [Leu]enkephalin on the GPI and that a Dyn(1-13) analogue containing the cyclic enkephalin segment, [D-Cys$^2$-Cys$^5$]Dyn(1-13), was 5-fold more potent than Dyn(1-13) on the GPI.[26] In addition, Shearman et al. revealed that [D-Cys$^2$-Cys$^5$]Dyn(1-13) displayed almost no $\kappa$-receptor activity but high $\delta$-receptor activity instead.[34] We applied this modification to our Dyn(1-8)-$NH_2$ ana-

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logue, resulting in potent activity. Surprisingly, [D-Cys$^2$-Cys$^5$,MeArg$^7$,D-Leu$^8$]Dyn(1–8)-NHEt (**20**) showed 40–60-fold more potent opioid activity than those of **18** in the three bioassays and its analgesic activity was 3.4-fold more potent than that of **18**. To our knowledge **20** is more potent than any other analogues of Dyn and its fragments so far reported. In the binding assays, however, **20**, as compared with **18**, showed about 5-fold higher $\mu$-receptor affinity and 17-fold higher $\delta$-receptor affinity but 1.6-fold lower $\kappa$-receptor affinity, indicating that **20** no longer has $\kappa$-receptor selectivity. This higher opioid activity observed in the bioassays than was expected on the basis of the results obtained in the binding assays could be partly explained by enhanced intrinsic activity of **20** at the receptors. Similar phenomenon had been observed with other cyclic opioid peptides.[18,35,36]

RVD has been considered to have only the $\kappa$ receptor, since $\kappa$-receptor agonists such as ethylketocyclazocine and bremazocine inhibit its contraction, whereas $\mu$-, $\delta$-, $\sigma$-, and $\epsilon$-receptor agonists, such as morphine, FK33-824, enkephalin, [D-Ala$^2$,D-Leu$^5$]enkephalin, SKF-10,047, and $\beta$-endorphin have no effect.[23] On the other hand, there is increasing evidence in favor of heterogeneity of $\kappa$ receptors.[37–41] In addition, it has been reported that Dyn binds to both $\kappa_1$ and $\kappa_2$ receptors but that U69,593 is specific for the $\kappa_1$ receptor (inactive at the $\kappa_2$ receptor).[41] Therfore, as one possible explanation of the striking discrepancy between the receptor selectivity observed in the bioassays and binding assays, it can be assumed that **20**, as compared with **18**, binds with much higher affinity to the $\kappa$-receptor subtype (e.g. $\kappa_2$ receptor) with which [$^3$H]U69,593 does not interact preferentially. Similarly in the case of such analogues as **3**, the discrepancy between the bioassay and binding assay data may be partly explained by this hypothesis. The results obtained in this study indicate that various probes are necessary to elucidate the receptor selectivity of opioid peptides. More studies are needed to confirm the opioid receptor selectivity of our analogues.

On the basis of the present results it is clear that the modification of the enkephalin segment to give metabolically stable analogues with high affinity and selectivity for the $\kappa$ receptor is strictly limited and that introduction of MeArg in position 7 protects the peptide effectively from enzymatic degradation without producing a major conformational change of the peptide and steric interference at the receptor. To our knowledge analogue **18** represents the first analogue of Dyn fragment that not only shows high affinity and relatively high selectivity for the $\kappa$ receptor but also produces a potent analgesic effect even when administered systemically into mice. Further studies are now in progress to investigate the pharmacological properties of **18**.

## Experimental Section

**General Methods.** Melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. Optical rotations were measured with a JASCO Model DIP-140 polarimeter. Amino acid analyses were carried out on a Hitachi 835 amino acid analyzer. Molecular weights of the products were determined by FAB-MS on JEOL Model JMS-HX100 mass spectrometer. Analytical and preparative HPLC was performed with an ALTEX Model 110A pump and a JASCO Model UVIDEC 100A ultraviolet detector. A Nucleosil 5C18 column (4.5 × 150 mm) was used in the analytical HPLC. TLC was performed on precoated silica gel plates (60F254, Merck) with use of the following solvent systems (all v/v): (A) AcOEt, (B) MeOH/$CHCl_3$ (1:7), (C) MeOH/AcOH/$CHCl_3$ (4:1:12), (D) $CHCl_3$/AcOEt (2:1).

A mixed anhydride was prepared as follows: Ethyl chloroformate (1 equiv) was added at about −20 °C to a solution of a carboxy component (1 equiv) and NMM (1 equiv) in THF or DMF. The mixture was stirred for 5 min and a solution of an amine component was added.

Symbols and abbreviations are in accordance with recommendations of the IUPAC–IUB Joint Commission on Biochemical Nomenclature: *Biochem. J.* **1984**, *219*, 345. The following other abbreviations were used: MeTyr, *N*-methyltyrosine; Sar, *N*-methylglycine; Azgly, -NHNHCO-; Phe(*p*-$NO_2$), *p*-nitrophenylalanine; MePhe, *N*-methylphenylalanine; MeLeu, *N*-methylleucine; homoArg, homoarginine; MeArg, $N^{\alpha}$-methylarginine; Boc, *tert*-butoxycarbonyl; Z, benzyloxycarbonyl; $Cl_2$Bzl, 2,6-dichlorobenzyl; MBzl, 4-methylbenzyl; Tos, tosyl; TFA, trifluoroacetic acid; NMM, *N*-methylmorpholine; DCC, dicyclohexylcarbodiimide; HOSu, N-hydroxysuccinimide; HOBt, *N*-hydroxybenzotriazole; THF, tetrahydrofuran; DMF, dimethylformamide; TEA, triethylamine; DCU, *N,N'*-dicyclohexylurea.

**Boc-D-Leu-NHEt (21).** Seventy percent aqueous ethylamine (12.9 g, 0.2 mol) was added to a mixed anhydride prepared from Boc-D-Leu-OH·$H_2O$ (25 g, 0.1 mol) in THF (200 mL). After the mixture was stirred at 0 °C for 2 h, the solvent was evaporated off. The residue was dissolved in AcOEt and the solution was washed with 5% $NaHCO_3$ and water and concentrated to dryness (24.5 g, 95%): mp 103–106 °C; $[\alpha]^{20}_D$ +20.0° (*c* 1.0, MeOH); TLC $R_f$ (A) 0.77. Anal. ($C_{13}H_{26}N_2O_3$) C, H, N.

**Z-MeArg(Tos)-D-Leu-NHEt (22).** TFA·H-D-Leu-NHEt (813 mg, 3 mmol), prepared by treating compound **21** with TFA/anisole, was dissolved in THF (7 mL) and neutralized with TEA. This solution was added to a mixed anhydride prepared from Z-MeArg(Tos)-OH (1.43 g, 3 mmol) in THF (15 mL). After the mixture was stirred at 0 °C for 2 h, the solvent was evaporated off. The residue was dissolved in AcOEt and the solution was washed with 5% $NaHCO_3$ and water and concentrated to dryness (1.58 g, 85%): $[\alpha]^{20}_D$ 0 ± 0.5° (*c* 1.0, MeOH); TLC $R_f$ (B) 0.68. Anal. ($C_{30}H_{44}N_6O_6S$) C, H, N.

**Boc-Arg(Tos)-MeArg(Tos)-D-Leu-NHEt (23).** MeArg(Tos)-D-Leu-NHEt (1.1 g, 2.29 mmol), prepared by catalytic hydrogenation (10% Pd/C) of compound **22** at atmospheric pressure, and Boc-Arg(Tos)-OH (983 mg, 2.29 mmol) were dissoved in DMF (4 mL). To this solution were added HOBt (372 mg, 2.75 mmol) and DCC (520 mg, 2.52 mmol) at 0 °C. After the mixture was stirred at 4 °C overnight and then at room temperature for 30 h, the precipitated DCU was filtered off and the solvent was removed in vacuo. The residue was purified by column chromatography on silica gel ($CHCl_3$/MeOH, 15:1) (1.2 g, 59%): $[\alpha]^{20}_D$ −20.6° (*c* 1.0, MeOH); TLC $R_f$ (B) 0.64. Anal. ($C_{40}H_{64}N_{10}O_9S_2$·$H_2O$) C, H, N.

**Boc-Leu-Arg(Tos)-MeArg(Tos)-D-Leu-NHEt (24).** TFA·H-Arg(Tos)-MeArg(Tos)-D-Leu-NHEt (4.986 g, 5.5 mmol), prepared by treating compound **23** with TFA/anisole, was dissolved in DMF (12 mL) containing NMM (0.726 mL, 6.6 mmol). This solution was added to a mixed anhydride prepared from Boc-Leu-OH·$H_2O$ (1.645 g, 6.6 mmol) in DMF (12 mL). After the mixture was stirred at 0 °C for 2 h, the solvent was evaporated off and the residue was dissolved in AcOEt. The solution was washed with 5% $NaHCO_3$ and water, the AcOEt was removed in vacuo, and the residue was solidified with MeOH/ether (5.28 g, 95%): mp 120–125 °C dec; $[\alpha]^{20}_D$ −25.8° (*c* 1.0, MeOH); TLC $R_f$ (B) 0.66. Anal. ($C_{46}H_{75}N_{11}O_{10}S_2$·$CH_3OH$) C, H, N.

**Boc-Phe-Leu-Arg(Tos)-MeArg(Tos)-D-Leu-NHEt (25).** TFA·H-Leu-Arg(Tos)-MeArg(Tos)-D-Leu-NHEt (4.691 g, 4.6 mmol), prepared by treating compound **24** with TFA/anisole, was dissolved in DMF (12 mL) containing NMM (0.608 mL, 5.52 mmol). This solution was added to a mixed anhydride prepared from Boc-Phe-OH (1.465 g, 5.52 mmol) in DMF (12 mL). After

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the mixture was stirred at 0 °C for 2 h, the solvent was evaporated off and the residue was dissolved in AcOEt. The solution was washed with 5% $NaHCO_3$ and water, the AcOEt was removed in vacuo, and the residue was solidified with MeOH/ether (5.07 g, 96%): mp 127–132 °C dec; $[\alpha]^{20}_D$ –25.4° (c 1.0, MeOH); TLC $R_f$ (B) 0.66. Anal. ($C_{55}H_{84}N_{12}O_{11}S_2 \cdot CH_3OH$) C, H, N.

**Boc-MeTyr(Cl$_2$Bzl)-Gly-Gly-OH (26).** Boc-MeTyr-(Cl$_2$Bzl)-OH (9.09 g, 20 mmol) and HOSu (2.53 g, 22 mmol) were dissolved in THF (100 mL) and DCC (4.12 g, 20 mmol) was added at 0 °C. After the mixture was stirred at 4 °C overnight, the precipitated DCU was removed by filtration and a solution of H-Gly-Gly-OH (2.91 g, 22 mmol) and $NaHCO_3$ (1.848 g, 22 mmol) in water (38 mL) was added. After stirring at room temperature for 40 h, the solvent was evaporated off, and 10% citric acid and AcOEt were added to the residue. The AcOEt layer was separated and washed with water. The solvent was removed in vacuo and the residue was purified by column chromatography on silica gel ($CHCl_3$/MeOH, 30:1) (9.23 g, 81%): mp 70–80 °C dec; $[\alpha]^{20}_D$ –47° (c 1.0, MeOH); TLC $R_f$ (C) 0.79. Anal. ($C_{26}H_{31}N_3O_7Cl_2 \cdot {}^1/_2C_2H_5OC_2H_5$) C, H, N.

**Boc-MeTyr(Cl$_2$Bzl)-Gly-Gly-Phe-Leu-Arg(Tos)-MeArg-(Tos)-D-Leu-NHEt (27).** Compound **26** (682 mg, 1.2 mmol) and HOBt (195 mg, 1.44 mmol) were dissolved in DMF (4 mL), and DCC (272 mg, 1.32 mmol) was added at 0 °C. After stirring for 2 h, a solution of TFA·H-Phe-Leu-Arg(Tos)-MeArg(Tos)-D-Leu-NHEt (1.167 g, 1.0 mmol), prepared by treating compound **25** with TFA/anisole, and NMM (0.132 mL, 1.2 mmol) in DMF (8 mL) was added. The mixture was stirred at 4 °C overnight and then the precipitate was removed by filtration. The solvent was evaporated off and the residue was purified by column chromatography on silica gel ($CHCl_3$/MeOH, 20:1) (1.39 g, 72%): mp 130–135 °C dec; $[\alpha]^{20}_D$ –35.3° (c 1.0, MeOH); TLC $R_f$ (B) 0.64. Anal. ($C_{76}H_{105}N_{15}O_{15}S_2Cl_2 \cdot CH_3OH \cdot H_2O$) C, H, N.

**MeTyr-Gly-Gly-Phe-Leu-Arg-MeArg-D-Leu-NHEt (18).** Compound **27** (220 mg, 0.137 mmol) was treated for 1 h at –5 °C with anhydrous liquid HF (10 mL) in the presence of anisole (0.2 mL). After removal of the HF in vacuo, the residue was dissolved in water and the solution was treated with Amberlite IRA-93 (acetate form) and lyophilized. The crude product was purified by HPLC on Nucleosil 5C18 (2 × 25 cm) using $H_2O/CH_3CN$ (81:19) containing 0.1% HCl as an eluent (70 mg, 49%). Characterization of the product is summarized in Tables I and II.

**Boc-Cys(MBzl)-Arg(Tos)-MeArg(Tos)-D-Leu-NHEt (28).** TFA·H-Arg(Tos)-MeArg(Tos)-D-Leu-NHEt (1.813 g, 2 mmol) was dissolved in DMF (4 mL) containing NMM (0.264 mL, 2.4 mmol). This solution was added to a mixed anhydride prepared from Boc-Cys(MBzl)-OH (0.712 g, 2.2 mmol) in DMF (4 mL). After stirring at 0 °C for 2 h, the solvent was evaporated off. The residue was dissolved in AcOEt and the solution was washed with 5% $NaHCO_3$ and water and concentrated to dryness (2.0 g, 91%): $[\alpha]^{20}_D$ –20.7° (c 1.0, MeOH); TLC $R_f$ (B) 0.54. Anal. ($C_{51}H_{77}N_{11}O_{10}S_3 \cdot H_2O$) C, H, N.

**Boc-Phe-Cys(MBzl)-Arg(Tos)-MeArg(Tos)-D-Leu-NHEt (29).** TFA·H-Cys(MBzl)-Arg(Tos)-MeArg(Tos)-D-Leu-NHEt (1.616 g, 1.45 mmol), prepared by treating compound **28** with TFA/anisole, was dissolved in DMF (4 mL) containing NMM (0.191 mL, 1.74 mmol). This solution was added to a mixed anhydride prepared from Boc-Phe-OH (0.462 g, 1.74 mmol) in DMF (4 mL). After the mixture was stirred at 0 °C for 2 h, the solvent was evaporated off. The residue was dissolved in AcOEt and the solution was washed with 5% $NaHCO_3$ and water and concentrated to dryness (1.663 g, 92%): $[\alpha]^{20}_D$ –22.8° (c 1.0, MeOH); TLC $R_f$ (B) 0.54. Anal. ($C_{60}H_{86}N_{12}O_{11}S_3$) C, H, N.

**Boc-D-Cys(MBzl)-Gly-OEt (30).** A solution of HCl·H-Gly-OEt (1.396 g, 10 mmol) and NMM (1.1 mL, 10 mmol) in DMF (20 mL) was added to a mixed anhydride prepared from Boc-D-Cys(MBzl)-OH (3.233 g, 10 mmol) in DMF (15 mL). After the mixture was stirred at 0 °C for 2 h, the solvent was evaporated off and the residue was dissolved in AcOEt. The solution was washed with 5% $NaHCO_3$ and water, the AcOEt was removed in vacuo, and the residue was solidified with *n*-hexane (3.6 g, 88%): mp 80–82 °C; $[\alpha]^{20}_D$ +30.2° (c 1.0, MeOH); TLC $R_f$ (D) 0.74. Anal. ($C_{20}H_{30}N_2O_5S$) C, H, N.

**Boc-Tyr(Cl$_2$Bzl)-D-Cys(MBzl)-Gly-OEt (31).** TFA·H-D-Cys(MBzl)-Gly-OEt (1.94 g, 4.27 mmol), prepared by treating compound **30** with TFA/anisole, was dissolved in THF (20 mL) containing TEA (1 mL, 7.17 mmol). This solution was added to a mixed anhydride prepared from Boc-Tyr(Cl$_2$Bzl)-OH (2.068 g, 4.697 mmol) in THF (20 mL). After the mixture was stirred at 0 °C for 2 h, the solvent was evaporated off. Water was added and the resulting precipitate was collected and recrystallized from MeOH/ether (2.66 g, 85%): mp 149–150 °C; $[\alpha]^{20}_D$ +17.0° (c 1.0, DMF); TLC $R_f$ (D) 0.63. Anal. ($C_{36}H_{43}N_3O_7SCl_2$) C, H, N.

**Boc-Tyr(Cl$_2$Bzl)-D-Cys(MBzl)-Gly-OH (32).** NaOH (1 N, 3.2 mL) was added to a solution of compound **31** (2.345 g, 3.2 mmol) in THF (30 mL). The mixture was stirred at room temperature for 1 h and then neutralized with 1 N HCl (3.2 mL). The solvent was evaporated off and the residue was solidified with water (1.899 g, 84%): mp 133–138 °C; $[\alpha]^{20}_D$ +35.8° (c 1.0, MeOH); TLC $R_f$ (B) 0.25. Anal. ($C_{34}H_{39}N_3O_7SCl_2$) C, H, N.

**Boc-Tyr(Cl$_2$Bzl)-D-Cys(MBzl)-Gly-Phe-Cys(MBzl)-Arg-(Tos)-MeArg(Tos)-D-Leu-NHEt (33).** Compound **32** (321 mg, 0.456 mmol) and HOBt (74 mg, 0.547 mmol) were dissolved in DMF (1 mL), and DCC (104 mg, 0.502 mmol) was added at 0 °C. After the mixture was stirred for 2 h, a solution of TFA·H-Phe-Cys(MBzl)-Arg(Tos)-MeArg(Tos)-D-Leu-NHEt (475 mg, 0.38 mmol), prepared by treating compound **29** with TFA/anisole, and NMM (0.050 mL, 0.456 mmol) in DMF (2 mL) was added and the mixture was left to stand overnight at 4 °C. The solvent was evaporated off and the residue was purified by column chromatography on silica gel ($CHCl_3$/MeOH, 50:1) (380 mg, 55%): $[\alpha]^{20}_D$ –20.7° (c 0.5, DMF); TLC, $R_f$ (B) 0.61. Anal. ($C_{89}H_{115}N_{15}O_{15}S_4Cl_2 \cdot H_2O$) C, H, N.

**Tyr-D-Cys-Gly-Phe-Cys-Arg-MeArg-D-Leu-NHEt (20).** Compound **33** (300 mg, 0.164 mmol) was treated for 1 h at –5 °C with anhydrous liquid HF (10 mL) in the presence of anisole (0.2 mL). After removal of the HF in vacuo, the residue was dissolved in water and the solution was treated with Amberlite IRA-93 (acetate form) and lyophilized. The free peptide thus obtained was dissolved in water (700 mL) and the solution was adjusted to pH 8 with aqueous $NH_3$. After air was introduced therein with stirring for 2 days, the solution was readjusted to pH 6 and lyophilized. The crude product was purified by HPLC on Nucleosil 5C18 (2 × 25 cm) using $H_2O/CH_3CN$ (84:16) containing 0.05% HCl as an eluent (70 mg, 40%). Characterization of the product is summarized in Tables I and II.

**Bioassays and Analgesic Tests.** The bioassays (GPI, MVD, and RVD) and the tail-pinch tests were performed by the methods described elsewhere.[21-24]

**Binding Assays.** Crude $P_2$ membrane fraction prepared from male Hartley guinea pig brains was used. The membrane fraction suspended in 50 mM Tris-HCl (pH 7.6) at a concentration of 1 g original wet weight/20 mL was incubated at 25 °C for 60 min with a radiolabeled ligand ([$^3$H]DAGO, 5 nM; [$^3$H]U-69,593, 10 nM; [$^3$H]DPDPE, 5 nM) and various concentrations of compounds. Incubation was terminated by the rapid filtration of the mixture through whatman GF/B filter presoaked in 1% polyethylenimine, and the filters were rinsed six times with ice-cold 50 mM Tris-HCl (pH 7.6). The radioactivity retained on the filter was counted by liquid scintillation counter. Nonspecific binding was determined by the addition of 10 μM levallorphan. The inhibition constant ($K_i$) of the compound was calculated according to the following equation: $K_i = IC_{50}/(1 + [L]/K_D)$, where [L] is a ligand concentration described above. Dissociation constants ($K_D$) of the ligand which were determined by increasing the ligand concentrations were 3.27, 12.585, and 5.090 for [$^3$H]DAGO, [$^3$H]U-69,593, and [$^3$H]DPDPE, respectively.

**Registry No.** 1, 103614-11-5; **2**, 103614-19-3; **3**, 122623-86-3; 4, 103614-20-6; **5**, 122623-87-4; **6**, 122623-88-5; **7**, 103614-14-8; **8**, 103614-16-0; **9**, 103614-18-2; **10**, 122623-89-6; 11, 103614-17-1; **12**, 122623-90-9; **13**, 103638-93-3; 14, 103638-94-4; **15**, 103614-21-7; **16**, 103614-12-6; **17**, 103614-13-7; **18**, 103613-84-9; **19**, 103614-23-9; **20**, 103614-26-2; **21**, 103613-68-9; **22**, 103613-69-0; **23**, 103613-72-5; **24**, 103613-75-8; **25**, 103613-78-1; **26**, 103613-80-5; **27**, 103613-83-8; **28**, 122623-91-0; **29**, 122623-92-1; **30**, 103613-98-5; **31**, 103614-01-3; **32**, 103614-02-4; **33**, 122647-82-9; BOC-D-Leu-OH, 16937-99-8; $EtNH_2$, 75-04-7; H-D-Leu-NHEt·TFA, 103613-71-4; Z-MeArg-(Tos)-OH, 62937-37-5; H-MeArg(Tos)-D-Leu-NHEt, 103638-92-2; BOC-Arg(Tos)-OH, 13836-37-8; H-Arg(Tos)-MeArg(Tos)-D-Leu-NHEt·TFA, 103613-74-7; BOC-Leu-OH, 13139-15-6; H-Leu-Arg(Tos)-MeArg(Tos)-D-Leu-NHEt·TFA, 103613-77-0; BOC-

Phe-OH, 13734-34-4; BOC-MeTyr($Cl_2$Bzl)-OH, 57817-43-3; H-Gly-Gly-OH, 556-50-3; H-Phe-Leu-Arg(Tos)-MeArg(Tos)-D-Leu-NHEt·TFA, 103613-82-7; BOC-Cys(MBzl)-OH, 61925-77-7; H-Cys(MBzl)-Arg(Tos)-MeArg(Tos)-D-Leu-NHEt·TFA, 122623-94-3; BOC-D-Cys(MBzl)-OH, 61925-78-8; H-Gly-OEt·HCl, 623-33-6; H-D-Cys(MBzl)-Gly-OEt·TFA, 103614-00-2; H-Phe-Cys-(MBzl)-Arg(Tos)-MeArg(Tos)-D-Leu-NHEt·TFA, 122623-96-5; H·Tyr-D-Cys-Gly-Phe-Cys-Arg-MeArg-D-Leu-NHEt, 122623-97-6.

# Synthesis and Antifolate Properties of 9-Alkyl-10-deazaminopterins

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Reformatski condensation of benzyl 2-bromopropionate with 4-carbomethoxybenzaldehyde, followed by dehydration afforded benzyl 2-methyl-*p*-carbomethoxycinnamate (**4a**). Hydrogenation over a Pd catalyst gave the hydrocinnamic acid **5a**. Conversion to the chloromethyl (**6a**) and azidomethyl ketone (**7a**) was followed by hydrogenation to the aminomethyl ketone (**8a**). Direct N-alkylation by 2,4-diamino-5-nitro-6-chloropyrimidine followed by reductive ring closure in Zn–HOAc and subsequent saponification of the benzoate ester yielded 4-amino-4-deoxy-9-methyl-10-deazapteroic acid (**11a**). Coupling with diethyl L-glutamate and saponification afforded 9-methyl-10-deazaminopterin (**13a**). The 9-ethyl analogue (**13b**) was similarly prepared from benzyl 2-bromobutyrate. The 9-methyl analogue (**13a**) was 21 times more potent than MTX as an inhibitor of cell growth in L1210 cells. The reason for this enhanced cytotoxicity in L1210 is unclear, since enzyme inhibition and transport parameters were similar to those of MTX. In human Manca leukemia cells growth inhibition was not dramatic and paralleled MTX.

Past communications[1,2] from this laboratory have documented the synthesis and selective antitumor activity of 10-alkyl-10-deazaminopterin compounds. These deazapteridine analogues of methotrexate (MTX) were found to have enhanced transport into tumor tissues and were sparing of sensitive host epithelial and bone marrow cells. Such modification gave analogues with much improved therapeutic indices compared with MTX, and one compound, 10-ethyl-10-deazaminopterin, has undergone extensive clinical trials with favorable results.[3,4] It was also of interest to determine whether the 10-position variants were uniquely responsible for the selective transport into tumor cells. It was noted in early studies with antifolate compounds that 9-methylaminopterin[5,6] showed activity approximating that of MTX in bacterial and mouse tumor model assays. In this paper we report studies with 9-alkyl-10-deazaminopterin analogues.

## Chemistry

The synthesis of 9-methyl-10-deazaminopterin (**13a**) began with 2-bromopropionic acid (**1a**) (Scheme I). This acid was converted to its benzyl ester (**2a**) by treatment with benzyl alcohol catalyzed by *p*-toluenesulfonic acid in benzene.[7] Attempts to convert **2a** to a dimethyl α-phosphono ester gave low yields of product with scrambled alkoxy groups. We thus abandoned plans for an Emmons–Horner condensation with *p*-carbomethoxybenzaldehyde and resorted to Reformatski condensation of **2a** with the aldehyde as promoted by Zn dust in benzene. Instead of directly yielding the expected cinnamate ester (**4a**), the reaction afforded the tertiary alcohol ester **3a**. This carbinol was resistant to direct dehydration, but was converted to **4a** by treatment with $POCl_3$ followed by dehydrohalogenation of the chloro intermediate with diazabicyclononene in 61% yield from **3a**. The benzyl 2-methyl-*p*-carbomethoxy intermediate **4a** was hydrogenated over Pd black at ordinary pressure to saturate the olefinic bond with concomitant hydrogenolysis of the benzyl ester to give the 2-methyl-*p*-carbomethoxyhydrocinnamic acid **5a** in 87% yield.

Table I. Cell Growth, Enzyme Inhibition, and Transport Properties for L1210[a]

| compd | L1210 growth inhibn $IC_{50}$, nM | L1210 DHFR inhibn $K_i$, nM | influx[b] $K_i$, μM |
|---|---|---|---|
| **13a** | 0.24 | 0.0052 | 3.0 |
| **13b** | 4.7 | 0.0055 | 8.2 |
| 10-DA | 1.5 | 0.0034 | 1.2 |
| 10-Et-10-DA[c] | 0.65 | 0.0028 | 0.99 |
| MTX | 5.0 | 0.0056 | 3.9 |

[a] See ref 1 for methods. [b] Expressed in terms of competition with [$^3$H]MTX binding, thus a measure of binding to the cellular enzyme responsible for active transport of folates (see ref 11). [c] Data from ref 11.

Acid **5a** was converted to its acid chloride ($SOCl_2$) followed by treatment with $CH_2N_2$ and HCl gas to afford the chloromethyl ketone **6a** in 79% yield. Treatment of **6a** with $NaN_3$ in 80% MeOH gave the azido ketone **7a** (94% yield), which was subsequently hydrogenated (Pd) to the amino ketone **8a**. Past experience[8] has required that the ketone function be blocked as the semicarbazone or ketal before alkylation of 2,4-diamino-5-nitro-6-chloropyrimidine in the Boon–Leigh type[9] of pteridine ring construction.

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