A Quantitative Structure–Activity Relationship Study of the Inhibitory Action of a Series of Enkephalin-like Peptides in the Guinea Pig Ileum and Mouse Vas Deferens Bioassays

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The potencies of a series of enkephalin derivatives with general structure H·Tyr-D-Ala-Gly-X-Y·NH_2 (X and Y are variable amino acid residues), to depress the contractions of electrically stimulated guinea pig ileum and mouse vas deferens preparations, were analyzed. The doses for half-maximal inhibition could be expressed as linear functions of three structural parameters—electronic, hydrophobic, and steric—which characterized the side chains of X and Y. A general methodology was devised for the quantitative estimation of these substituent parameters for amino acid side chains. The excellent statistics obtained for the equation of regression is an indication that no other parameters need to be considered to account for the opiate activity in this series. The relative importance of these factors and their intercorrelation were established, and the predictive value of the model was tested.

The natural enkephalins Tyr-Gly-Gly-Phe-Leu and Tyr-Gly-Gly-Phe-Met[1] are endogenous peptides that interact with opiate receptors in much the same way as morphine. The organization of the information in these linear pentapeptides appears to be of both the rhegnylogic (three-dimensional, conveyed by elements that are neighbors in the folded structure but dispersed in the peptide sequence) and sychnologic (one-dimensional, contained in a flexible segment of adjacent amino acids) types (for a detailed description of these concepts, see, e.g., ref 2). On the one hand, the conformation of the enkephalins shown to exist in the crystalline state[3] is also likely to be preferred in solution.[4] It is characterized by a β bend and involves two hydrogen bonds between tyrosine and phenylalanine. This folded structure resembles morphine and probably requires only minor adjustments for optimal recognition of the receptor. On the other hand, the segment made of the three N-terminal residues can be identified as the hormonal message[2] or at least as its main part, while the residues in positions 4 and 5 serve as auxiliary elements (potentiator and address[2]) optimizing the receptor interaction with the message sequence and influencing the selectivity toward receptor subclasses.[5] The fact that neither the natural tripeptide segment Tyr-Gly-Gly nor any of its synthetic modifications display opiate activity indicates that the presence of a fourth residue is mandatory for the message to be complete. However, depending on the nature of its side chain, considerable modulation of the potency (potentiator) and selectivity (address) is observed. Thus, even though the rhegnylogic and sychnologic concepts appear to overlap in these short polypeptides, they do, however, both focus on the N-terminal tetrapeptide as the part triggering the opiate receptor. An interesting fact is the possible replacement of glycine-2 in this segment by D residues, such as D-alanine, D-methionine, or D-serine, but not by L residues, without loss of activity. This result, confirmed in a large number of synthetic analogues, for example,[6] is explained by the impossibility for L amino acids to adopt the conformation of glycine-2 which prevails in crystalline leucine-enkephalin.

Table I. Hydrophobic (π), Electronic (S), and Steric (ν_{CH} and ν) Parameters for the Side Chain of Amino Acids Involved in the QSAR Analysis

amino acid	π	S	ν_{CH}	ν
adamantylalanine	3.24	3.04	1.43	9.30
tert-butylglycine	1.51		1.24	4.00
carboranylalanine	4.20	−1.28	1.38	7.81
cyclohexylalanine	2.72	1.13	0.97	6.43
leucine	1.64		0.98	4.00
methionine	1.42		0.68	4.43
neopentylglycine	1.89		1.34	5.00
p-nitrophenylalanine	1.96	−0.33	0.75	7.33
phenylalanine	1.63	0.43	0.70	5.88

Considerable attention was paid in our laboratory to the variable side chains of the residues in positions 4 and 5, as a means of modulating pharmacological potency and receptor subclass specificity. One main trait of our methodology was the introduction of artificial amino acids in which one or two structural properties, such as hydrophobicity, electronic, or steric factors, are stressed; in this way, we hoped to better elucidate their individual role in generating the biologically active conformation. Thus, a series of enkephalin peptides containing at least one of the residues carboranylalanine, adamantylalanine, *tert*-butylglycine, neopentylglycine, and *p*-nitrophenylalanine (Figure 1) has been synthesized chemically[8-11] and tested biochemically and pharmacologically.[5,12] It was therefore tempting to try to relate quantitatively their binding and inhibiting potencies to their structural features. The purpose of this work was to consider a series of congeners for which homogeneous measurements existed and to investigate the quantitative relationships between structure and biological response in at least one test for opiate activity. The present QSAR study analyzes the inhibiting potency of 12 enkephalin amides to depress the field-

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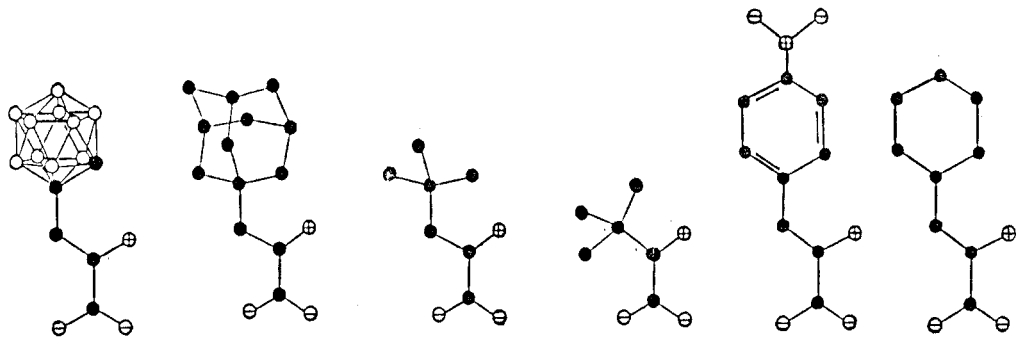

Figure 1. Schematic representation (from left to right) of (*S*)-(−)-2-amino-3-(*o*-carboranyl)propionic acid (carboranylalanine), (*S*)-(+)-2-amino-3-(1-adamantyl)propionic acid (adamantylalanine), (*S*)-(+)-2-amino-4,4′-dimethylpentanoic acid (neopentylglycine), (*S*)-(−)-2-amino-3,3′-dimethylbutanoic acid (*tert*-butylglycine), (*S*)-(−)-2-amino-3-(4-nitrophenyl)propionic acid (*p*-nitrophenylalanine), and (*S*)-(+)-2-amino-3-cyclohexylpropionic acid (cyclohexylalanine). The hydrogen atoms are omitted: (●) carbon, (○) boron, (⊕) nitrogen, (⊖) oxygen.

Table II. Input Values of the Structural Parameters and Experimental and Calculated Potencies of the Enkephalin Analogues on the Guinea Pig Ileum

no.	-X-Y-[a]	exptl[b] log $1/IC_{50}$ ± SEM	$\pi_X + \pi_Y$	S_X	$\nu_X + \nu_Y$	calcd log $1/IC_{50}$	Δ[c]
1	-phenylalanyl-leucyl-	7.95 ± 0.07	3.27	0.43	9.88	7.00	0.05
2	-phenylalanyl-methionyl-	7.94 ± 0.13	3.05	0.43	10.31	8.05	0.11
3	-phenylalanyl-adamantylalanyl-	8.22 ± 0.88	4.87	0.43	15.18	8.01	0.21
4	-phenylalanyl-*tert*-butylglycyl-	7.93 ± 0.13	3.14	0.43	9.88	7.95	0.02
5	-*p*-nitrophenylalanyl-leucyl-	8.50 ± 0.23	3.60	−0.33	11.33	8.50	0.01
6	-*p*-nitrophenylalanyl-methionyl-	8.64 ± 0.12	3.38	−0.33	11.76	8.65	0.01
7	-*p*-nitrophenylalanyl-adamantylalanyl-	8.42 ± 0.01	5.20	−0.33	16.63	8.62	0.20
8	-*p*-nitrophenylalanyl-neopentylglycyl-	8.72 ± 0.22	3.85	−0.33	12.33	8.55	0.17
9	-carboranylalanyl-leucyl-	8.33 ± 0.09	5.84	−1.28	11.81	8.31	0.02
10	-carboranylalanyl-methionyl-	8.44 ± 0.22	5.62	−1.28	12.24	8.46	0.02
11	-cyclohexylalanyl-leucyl-	7.04 ± 0.23[d]	4.36	1.13	10.43	7.05	0.01
12	-cyclohexylalanyl-methionyl-	7.14 ± 0.23[d]	4.14	1.13	10.86	7.20	0.06

[a] The complete structure of each congener is H·Tyr·D-Ala·Gly-X-Y·NH_2. [b] IC_{50} = molar concentration of analogue for 50% inhibition. [c] Δ = deviations of the calculated from the experimental values. [d] Calculated from ref 6.

stimulated contractions of the myenteric plexus longitudinal muscle preparation of the guinea pig ileum and of 8 analogues to depress those of the mouse vas deferens.

Methods

The pharmacological bioassays on the two isolated tissues have been described.[28,29] The pentapeptides analyzed were prepared in our laboratory by classical methods of peptide synthesis in solution.[13] For reasons given in the beginning of this paper, they had the general structure H·Tyr-D-Ala-Gly-X-Y·NH_2, where X and Y are variable amino acid residues (Table II). The biological data were means of at least three experiments performed in the same laboratory (P. W. Schiller, Clinical Research Institute of Montreal) under the same conditions. Two exceptions were the potencies of compounds **11** and **12**, which are literature values.[6] The familiar form log $1/IC_{50}$ was chosen, where IC_{50} is the concentration of hormone analogue producing 50% of the maximmal response. Biological activity varied from 7.04 for one of the weakly active analogues containing cyclohexylalanine (Figure 1) in position 4 to 8.72 for the approximately 50 times more potent *p*-nitrophenylalanine (4), neopentylglycine (5) analogue (Table II). The SEM did not exceed 0.25 log unit. The potency of the reference compound, methionine-enkephalin (which was not a member of the series analyzed), amounts to 7.42 on this scale. Regression analysis of the data was performed by a classical computing program[14] which also provided the statistical examination of the fit. The linear free energy related model (eq 1) was

$$\log 1/IC_{50} = k_0 + \sum_{i=1}^{n} k_i P_i \qquad (1)$$

used, in which P_i is the physicochemical parameter of the side chains of X and Y, k_0 and k_i are the regression constants, and n is the number of parameters or parameter combinations involved. Since the latter characterizes two different positions of the pentapeptide, the model implies additivity of the contributions from X and Y. The validity of this assumption was checked by a Free–Wilson[21] analysis of the data of Table II.

Three kinds of side-chain parameters were considered. (1) Hydrophobicity (lipophilicity) was expressed by the Hansch substituent parameter π obtained for amino acid side chains by eq 2, in which P is the partition coefficient of the free amino acid

$$\pi \text{ (side chain)} = \log P \text{ (amino acid)} - \log P \text{ (glycine)} \qquad (2)$$

in the 1-octanol/water system at neutral pH.[15] From Table I, it can be seen that carboranylalanine and adamantylalanine display extremely high lipophilicities.[16] (2) A measure of the electronic properties of amino acid side chains was found by postulating that the pK_a of any carboxylic acid (R–COOH) reflects the electronic features of R. A substituent parameter S was obtained by constructing, analogously to eq 2, eq 3. However,

$$S = pK_a \text{ (R-COOH)} - pK_a \text{ (HCOOH)} \qquad (3)$$

it was found convenient to consider R as the substituent of alanine-C_β (instead of glycine-C_α), since R could generally be assigned to a ring structure (phenyl, nitrophenyl, carboranyl, cyclohexyl, and adamantyl) for which the corresponding pK_a was known.[17-19]

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No S value can be assigned by this method to the side chains of proline, valine, or *tert*-butylglycine. It can be seen from Table II that a broad range of S values was covered in the analogues tested. (3) An excellent estimation of the bulk of a substituent is given by Charton's steric parameter ν_{CH}, the value of which is known for a large number of groups.[20] This parameter, which is related to Taft's constant E_s, is essentially a measure of the steric hindrance by the substituent X of the hydrolysis of the ester X-CH_2COOH. However, it is expressed in terms of the minimal van der Waals radius r_{min} and defines as a substituent parameter, e.g., for a CR_3 group, as

$$\nu_{CH}(CR_3) = r_{min}(CR_3) - r(H) \quad (4)$$

$$r(H) = 1.24 \text{ Å}$$

Although very convenient, Charton's values do in fact express the steric bulk as seen from the reaction center of hydrolysis in the model compounds. Thus, it can be seen, for example, that ν_{CH} for neopentyl (1.34) is very similar to ν_{CH} for adamantyl (1.43) despite the large difference in size of the two groups (Table I). In order to better describe the space-filling properties of amino acid side chains, as seen by the receptor sites, we chose the van der Waals volume instead, a parameter which can be measured directly, for example, on the CPK precision molecular models (Ealing Scientific Ltd., Cambridge, MA). A normalized substituent parameter, ν, was obtained with the formula $\nu(R) = [V(R) - V(H)]/V(CH_2)$, where V is the measured van der Waals volume. From this, $\nu = 1$ for the side chain of alanine, while the values for neopentylglycine and adamantylalanine are 5 and 9.3, respectively (Table I).

Regression was systematically applied to the experimental biological activities with one, two, three, or more structural parameters or combinations of them. Statistical analysis allowed the testing, in each case, of the correlation obtained. The criteria employed were the value of the multiple correlation coefficient r, the value of the overall F ratio, and the amount of explained variance EV. The individual regression coefficients were tested for significance by the Student's t test.

Results

The regression performed in the Free–Wilson analysis was significant at the 99% level according to an F test (F = 23.7, DF = 7 and 4). The multiple correlation coefficient was 0.988, and the amount of explained variance was 93.5%. These values confirmed the additivity of the contributions of the side chains of X and Y to the overall biological activity.

Examination of Table III, which reports the result of 14 regression analyses, shows that no statistically satisfying correlation was obtained with combinations of less than three parameters. The best fit with one single parameter was given by the electronic factor S_X, tending to prove its preponderant role for biological activity. The best correlation was obtained with the sum of the hydrophobic π's for the residues X and Y, the electronic factor S for X, and the sum of the steric ν's for X and Y (run 11). The inclusion of squared terms (run 14) did not improve the fit: the F ratio diminished and only one of the five t values was significant. The correlation given in eq 5 accounts

$$\log 1/IC_{50} = -0.411\ (\pm 0.13)\ (\pi_X + \pi_Y) - 0.694\ (\pm 0.13)\ S_X + 0.146\ (\pm 0.05)\ (\nu_X + \nu_Y) + 8.103 \quad (5)$$

$$n = 12,\ r = 0.979,\ s = 0.128,\ F = 62.8,\ EV = 0.941$$

accurately for the inhibiting activity of the 12 congeners, as confirmed by the statistical analysis. More than 99% significance is achieved in both the F and the t tests. Comparison of the calculated with the experimental activities (Table II) also shows that the deviations (Δ) are generally not higher than the SEM of the experimental data. Equation 5 predicts that an increase in hydrophobicity of either X or Y or an increase of the electronic factor (S) of X will lower the biological activity. The reverse is true for the steric components ν_X and ν_Y. The design of an optimal compound remains, however, problematic, as the factors are intercorrelated and cannot be varied independently: the elements of the correlation matrix were −0.31 for S_X and $(\nu_X + \nu_Y)$, −0.53 for S_X and $(\pi_X + \pi_Y)$, and 0.58 for $(\pi_X + \pi_Y)$ and $(\nu_X + \nu_Y)$, indicating a nonnegligible covariance of the latter two parameter combinations. When applied to the analogue [D-Ala^2,-adamantylalanyl^4]enkephalin amide not included in the series analyzed, eq 5 led to a calculated potency (5.93) that agreed well with the experimental one (6.00 ± 0.17). Clearly, if this calculation was performed at the right time, then the synthesis of this weakly active analogue would have been unnecessary.

Table III. Summary of Fourteen Analyses: the Best Correlation Is Obtained in Run 11, in Which All t Values Are Significant

run	parameters used	r^2[a]	explained variance	F[b]	DF[c]	t[d]
1	π_X	0.01	−0.09	0.01	1, 10	−0.08
2	π_Y	0.05	−0.04	0.55	1, 10	0.74
3	$\pi_X + \pi_Y$	0.01	−0.08	0.16	1, 10	0.40
4	S_X	0.63	0.59	17.28	1, 10	−4.15
5	ν_X	0.32	−0.25	4.66	1, 10	2.16
6	ν_Y	0.06	−0.04	0.58	1, 10	0.76
7	$\nu_X + \nu_Y$	0.19	0.11	2.34	1, 10	1.53
8	$\pi_X + \pi_Y$	0.75	0.70	13.99	2, 9	−2.13
	S_X					−5.23
9	S_X	0.67	0.59	9.21	2, 9	−3.63
	$\nu_X + \nu_Y$					1.05
10	$\pi_X + \pi_Y$	0.21	−0.04	1.23	2, 9	−0.53
	$\nu_X + \nu_Y$					1.50
11	$\pi_X + \pi_Y$	0.959	0.944	62.77	3, 8	−7.51
	S_X					−12.08
	$\nu_X + \nu_Y$					6.34
12	$(\pi_X + \pi_Y)^2$	0.83	0.77	13.46	3, 8	−1.97
	$\pi_X + \pi_Y$					1.77
	S_X					−5.90
13	$(\nu_X + \nu_Y)$	0.67	0.55	5.48	3, 8	0.14
	$\nu_X + \nu_Y$					0.20
	S_X					−2.80
14	$(\pi_X + \pi_Y)^2$	0.974	0.95	44.90	5, 6	0.02
	$\pi_X + \pi_Y$					−0.52
	S_X					−6.11
	$(\nu_X + \nu_Y)^2$					−1.39
	$\nu_X + \nu_Y$					1.73

[a] r^2 = squared multiple correlation coefficient. [b] F = variance ratio. [c] DF = degrees of freedom. [d] t = Student's ratio of a regression coefficient to its standard error.

Table IV. Experimental Potencies (Log $1/IC_{50}$ ± SEM) of Eight Analogues on the Guinea-Pig Ileum (GPI) and on the Mouse Vas Deferens (MVD)[a]

-X-Y-[a]	GPI	MVD
-phenylalanyl-leucyl-	7.95 ± 0.07	8.02 ± 0.07
-phenylalanyl-adamantylalanyl-	8.22 ± 0.08	7.30 ± 0.09
-phenylalanyl-*tert*-butylglycyl-	7.93 ± 0.13	8.01 ± 0.13
-*p*-nitrophenylalanyl-leucyl-	8.50 ± 0.23	8.41 ± 0.10
-*p*-nitrophenylalanyl-methionyl-	8.64 ± 0.12	8.50 ± 0.14
-*p*-nitrophenylalanyl-adamantylalanyl-	8.42 ± 0.01	7.69 ± 0.06
-carboranylalanyl-leucyl-	8.33 ± 0.98	7.48 ± 0.24
-adamantylalanyl-leucyl-	6.00 ± 0.17	5.53 ± 0.27

[a] The complete structure of each analogue was H·Tyr-D-Ala-Gly-X-Y·NH_2.

(20) M. Charton, in "Design of Biopharmaceutical Properties through Prodrug and Analogs", E. Roche, Ed., American Pharmaceutical Association, Washington, DC, 1977, pp 228–280.
(21) S. M. Free and J. M. Wilson, *J. Med. Chem.*, 7, 395 (1964).

The predictive value of the model was also tested by performing the regression with a subgroup of the analogues available. The general result for several different choices was that practically the same information could be obtained with only 6 analogues instead of 12, provided each of the four possible residues in position 4 was represented at least once. One typical result with compounds **1**, **2**, **7–9**, and **12** was eq 6. The regression was still significant at

$$\log 1/IC_{50} = -0.455\ (\pm 0.44)\ (\pi_X + \pi_Y) - 0.799\ (\pm 0.51)\ S_X + 0.119\ (\pm 0.15)\ (\nu_X + \nu_Y) + 8.587 \quad (6)$$

$$n = 6,\ r = 0.983,\ s = 0.157,\ F = 19.7,\ EV = 0.918$$

the 95% level in the F test and the 92% level in the t test.

It is generally accepted that the ratio of μ- to δ-receptor sites is markedly higher in the GPI than in the MVD preparations used in the bioassays.[22] In order to investigate whether the relative importance of the parameters is different when the analogues act on μ or δ receptors, the QSAR analyses that were performed with eight analogues for which measurements were available on both the GPI and the MVD (Table IV) were compared. The results on the GPI (eq 7) and on the MVD (eq 8) reveal only minor

$$\log 1/IC_{50} = -0.382\ (\pm 0.18)\ (\pi_X + \pi_Y) - 0.659\ (\pm 0.11)\ S_X + 0.129\ (\pm 0.08)(\nu_X + \nu_Y) + 8.186 \quad (7)$$

$$n = 8,\ r = 0.992,\ s = 0.134,\ F = 90.8,\ EV = 0.974$$

$$\log 1/IC_{50} = -0.667\ (\pm 0.07)\ (\pi_X + \pi_Y) - 0.629\ (\pm 0.04)\ S_X + 0.076\ (\pm 0.03)\ (\nu_X + \nu_Y) + 9.685 \quad (8)$$

$$n = 8,\ r = 0.998,\ s = 0.049,\ F = 845,\ EV = 0.997$$

differences in the regression coefficients, indicating similar structural requirements of the two receptor subsites (or similar μ/δ ratios in both tissues).

Discussion

While it is an accepted concept that biological activity is a function of the structure of the drug, the nature of this function may be very complex and is unknown in the general case. From polypeptide hormones, it is generally expected that they act via a singular conformation, either prefolded or induced by the receptor.[23] The ease with which this active conformation is reached depends essentially upon the primary structure of the polypeptide. As far as the dominating physicochemical parameters of the side chains that induce and promote the correct folding can be identified, biological activity is likely to be describable by a combination of these parameters.

Very few QSAR studies[24-26] of polypeptide hormones have appeared so far, and this is, to my knowledge, the first one dealing with the enkephalins. The methodology proposed in this work is simple and widely applicable, and it does not rely upon the intuition of the chemist. According to this methodology, multiple regression is performed on the largest possible number of parameters or parameter combinations for the side chains of the position(s) varied and the best correlation according to statistics selected.

The high amount of variance (94.1%) explained by the regression equation (eq 5) is strong evidence that the inhibitory potency in the series studied is controlled mainly by the electronic properties of the side chains in position 4 and by the hydrophobic and steric features of those in positions 4 and 5. The fact that no additional parameter, taking proteolytic degradation into account, had to be considered may be due to the probable high correlation of this factor with the steric bulk of the C-terminal residue.

Since, on the one hand, the relative contributions to the biological activity are not very different on the GPI and the MVD and since, on the other hand, pharmacological and biochemical tests[9,12] correlate well, eq 5 seems to represent more of an overall than a selective opiate activity. One major reason for the lack of selectivity of the compounds analyzed is the presence of a C-terminal amide known to cancel most of the δ-orientating effects in many pentapeptide enkephalin analogues.[27] However, provided experimental data for the displacement of selective tritiated ligands from subclasses of binding sites would be available, the approach used in this study could be of great help to identify the most relevant factors for μ, δ, or κ selectivity. The collection of these binding data and the investigation of their QSAR are now in progress.

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