Affinity of 1,2-substituted oxytocin analogues to the uterus receptor: Free-Wilson and Hansch analysis[1]

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Summary. The analysis of pA_2 values for 1,2-substituted oxytocin analogues suggests a significant resonance effect of p-substituted groups in 2-tyrosine when the hormone binds to its uterus receptor, whereas the N-terminal amino group exerts less clearly characterized effects (participation of its lipophilicity and molecular volume can be assumed).

Oxytocin analogues substituted at the p- and m-positions of 2-tyrosine and/or at the N-terminal amino group are known to be inhibitors of neurohypophyseal hormones[2]. With few exceptions, the inhibition measured in in vitro systems (isolated rat uterus, strip of mammary gland) is competitive. For isolated rat uterus, the measure of binding of a number of substances to the oxytocin receptor, the pA_2 value[3], was established. The question now arises as to whether structural requirements for binding of oxytocin to its uterine receptor can be deduced from these data.

The pA_2 values of such substances given in the literature, or calculated according to the published data are listed in the table, together with the varying structural features of their amino acid residues at positions 1 and 2 (figure 1). The 'additivity concept' for a series of biologically active compounds, first introduced by Free and Wilson[12], was used in the first step of the structure-binding analysis. This approach is briefly summarized as follows: Given a seris of biologically active compounds possessing the same basic structure but differing in substituents on several of its positions, the additivity concept assumes that the contribution of particular substituent on a particular position to the 'overall' biological activity is constant and independent of the substituents on the remaining positions. This contribution can be quantitatively expressed in the form of a substituent contant (designated 'additivity constant' or 'segment contribution') related to a particular position. The resulting biological activity of any substance from that series then equals the sum of a 'series mean value' ($\overline{pA_2}$ in our case, see below) plus all participating segment contributions. If biological activities of a group of substances with a sufficiently large number of substituent combinations are known, the segment contributions can be calculated - essentially by regression methods - and used, among other things for the design of new, biologically highly active substances. The mathematical background of this concept and the methodological restrictions have been described in sufficient details in the literature[13]. In a few cases, the Free-Wilson analysis has also been applied for biologically active peptides[14] where the amino acid residues at various positions can be considered as 'substituents'.

In our group of 1,2-substituted oxytocins, the segment contribution for the each substituent in the 2 positions was first calculated by a least squares optimalization procedure. In the following step, an attempt was made to combine the Free-Wilson and Hansch[15] methods. In the latter case, biological activities of compounds substituted in one particular position are correlated with parameters expressing physical properties of the substituent. These parameters are generally valid for any substitution with the corresponding group. Obviously it might be possible to search for correlations between segment contributions related to one particular position and the corresponding substituent constants. The following substituent constants were considered in the course of these investigations[16]: Hammett and Taft constant, σ_p and σ_m, for p- and m-substitution in position 2 (substituents R_2, R_3, figure 1); field and inductive constants, $\mathfrak{F}$, $\mathfrak{R}$, defined by Swain and Lupton[17], for N-terminal substitution (R_1); molar refraction, R_m, expressing the apparent molecular volume of the substituents R_1 and of the substituted phenyl ring in position 2 (i.e., $C_6H_3R_2R_3$, figure 1); and the Hansch lipophilicity constant, π, of the same substituents. Some of these constants are indicated in the table.

The calculations were impaired by 2 circumstances which might be critical in structure-activity studies in peptides: a) The data were taken from the literature and for this reason the substances do not represent an ideal systematic collection. Therefore, the Free-Wilson analysis could not be carried out fully. The cross-combinations of substituents

1,2-substituted analogues of oxytocin (OT): substituents, structural parameters (cf. fig. 1) and inhibition of oxytocin on rat uterus (pA_2-values)

No.	Compound	R_1	R_2	R_3	pA_2	Ref.	Substituent constants π for R_1	R_m for R_1	σ_p for R_2
1	[Phe^2]-OT	$-NH_2$	$-H$	$-H$	7.5	4	−1.23	5.42	0
2	[Phe(4-Me)^2]-OT	$-NH_2$	$-CH_3$	$-H$	8.0	4	−1.23	5.42	−0.17
3	[Phe(4-Et)^2]-OT	$-NH_2$	$-C_2H_5$	$-H$	7.7	4	−1.23	5.42	−0.15
4	[Tyr(Me)^2]-OT	$-NH_2$	$-OCH_3$	$-H$	7.8	4	−1.23	5.42	−0.27
5	[Tyr(Et)^2]-OT	$-NH_2$	$-OC_2H_5$	$-H$	7.5	4	−1.23	5.42	−0.25***
6	[Tyr(3-Me)^2]-OT	$-NH_2$	$-OH$	$-CH_3$	6.79	5	−1.23	5.42	−0.37
7	[Tyr(3-I)^2]-OT	$-NH_2$	$-OH$	$-I$	7.05	5	−1.23	5.42	−0.37
	deamino-[Phe(4-$NHCOCH_2Br$)^2]-OT	$-H$	$-NHCOCH_2Br$	$-H$	7.03	6	0	1.10	n.d.
	deamino-[Phe(4-$NHCOC_2H_5$)^2]-OT	$-H$	$-NHCOC_2H_5$	$-H$	6.95	6	0	1.10	−0.02***
10	N$^\alpha$-acetyl-[Tyr(Me)^2]-OT	$-NHCOCH_3$	$-OCH_3$	$-H$	7.13	7,8	−0.97	14.93	−0.27
11	N$^\alpha$-bromoacetyl-[Tyr(Me)^2]-OT	$-NHCOCH_2Br$	$-OCH_3$	$-H$	7.33	8	−0.21*	22.70	−0.27
12	N$^\alpha$-carbamoyl-[Tyr(Me)^2]-OT	$-NHCONH_2$	$-OCH_3$	$-H$	6.91	9	−1.30	13.72	−0.27
13	N$^\alpha$-mesyl-[Tyr(Me)^2]-OT	$-NHSO_2CH_3$	$-OCH_3$	$-H$	7.22	8	−1.18	18.17	−0.27
14	N$^\alpha$-maleoylglycyl-[Tyr(Me)^2]-OT	$-NHCOCH_2N$(CO−CH ‖ CO−CH)	$-OCH_3$	$-H$	7.27	8,10	−1.25**	33.20	−0.27
15	N$^\alpha$-glycyl-[Tyr(Me)^2]-OT	$-NHCOCH_2NH_2$	$-OCH_3$	$-H$	6.85	11	−2.53*	17.10	−0.27
16	N$^\alpha$-sarcosyl-[Tyr(Me)^2]-OT	$-NHCOCH_2NHCH_3$	$-OCH_3$	$-H$	6.52	11	−1.97*	21.72	−0.27
17	N$^\alpha$-pivaloyl-[Tyr(Me)^2]-OT	$-NHCOC(CH_3)_3$	$-OCH_3$	$-H$	7.26	11	0.48*	27.80	−0.27

*Calculated value; calculation based on additive properties of π-values; **calculated value; see reference 21; ***approximative value; estimate based on comparison of analogous pairs of substituents. n.d., not determined.

were mostly missing, and therefore only the minimal number of substances required for the calculations[13] was available. The calculated segment contributions (figure 2) are therefore somewhat problematic with regard to their general character and can by no means be used for any predictions. b) The published list of the substituent constants[16] is rather incomplete when applied to substitutions commonly used in peptides and proteins. In particular, many electronic substituent constants are missing. For this reason, only the first 13 substances listed in the table could be used for the analysis; even here, however, only approximate values of constants had to be employed for $\mathfrak{F}$ and σ_p in a few cases.

Nevertheless, this analysis indicates some interesting relations. To begin with, the segment contributions for substitution in position 2 (γ_2) correlate very significantly ($p < 0.05$) with a set of substituent constants if the correlation function

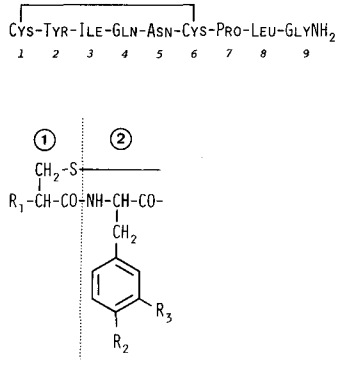

Fig. 1. Amino acid sequence of oxytocin (upper part) and the structural formula of its modified region (positions 1 and 2; lower part).

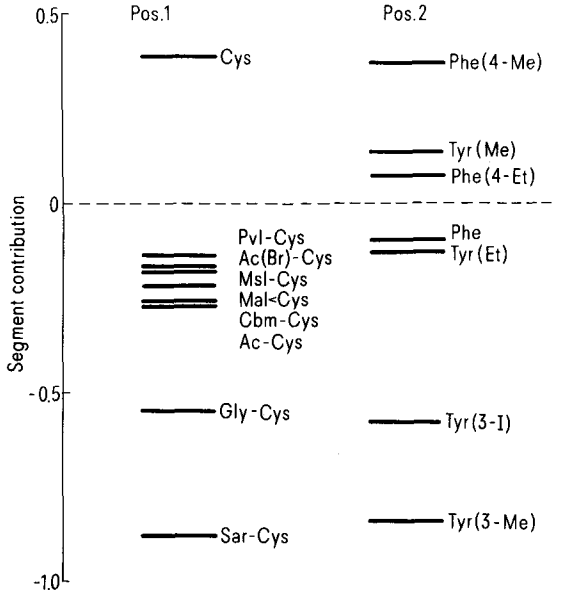

Fig. 2. Segment contributions in positions 1 and 2. Symbols according to the Recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (Biochim. biophys. Acta *263*, 205 (1972)). Nonlisted symbols: Ac(Br), N-bromoacetyl; Pvl, N-pivaloyl; Msl, N-mesyl; Cbm, N-carbamoyl (table).

includes the terms σ_p and σ_p^2; the simplest form of this function is then

$$\gamma_2 = a_0 + a_1 \sigma_p + a_2 \sigma_p^2 \quad (1)$$

(a_i are regression coefficients; coefficient of the multiple correlation is $r^2 = 0.853$). In fact, addition of any other term containing either substituent constant for position 2 does not result in a statistically significant increase of r^2. This increase was tested for its significance by an F-test procedure suggested for similar cases[18]. The test criterium is

$$F = ((r_1^2 - r_2^2)/(1 - r_1^2)) \cdot ((n - u_1 - 1)/(u_1 - u_2)) \quad (2)$$

where n is the total number of points, u is the number of independent variables in the correlation function; indices 1 and 2 relate to the correlation with higher and lower number of independent variables, respectively ($u_1 > u_2$); degrees of freedom are $\nu_1 = u_1 - u_2$ and $\nu_2 = n - u_1 - 1$.

Further, segment contributions for substitution at the N-terminus (γ_1) show some correlation with π, $\mathfrak{F}$ and R_m (linear terms; $r^2 = 0.950$; $p < 0.1$) but none of these substituent constants has dominating influence upon γ_1, as can be demonstrated by the F-test analysis mentioned above. The correlation function thus becomes

$$\gamma_1 = b_0 + b_1 \pi + b_2 \mathfrak{F} + b_3 R_m \quad (3)$$

(b_i are regression coefficients).

After these preliminary trials, the correlation analysis based on a general function

$$pA_2 = \overline{pA_2} + c_1 \gamma_1 + c_2 \gamma_2 \quad (4)$$

(c_1, c_2 are again regression coefficients) was carried out; for γ_1 and γ_2, single substituent constants were set in from equations (3) and (4), respectively. Several correlation functions which included combinations of linear and, in addition, quadratic terms were considered. If all 4 constants π, $\mathfrak{F}$, R_m for position 1, σ_p for position 2 are present in linear as well as in quadratic terms, the resulting correlation is very tight ($r^2 = 0.913$; $n = 13$) but its statistical significance is, due to a small number of points, rather low ($p > 0.1$). Moreover, the corresponding t-test procedure indicates an unambiguously insignificant influence of the terms $\mathfrak{F}$ and $\mathfrak{F}^2$. When omitted, a highly significant relation of the type

$$pA_2 = 7.26 - 3.48\,\pi - 1.83\,\pi^2 - 0.31\,R_m + 0.012\,R_m^2 - 5.46\,\sigma_p - 18.52\,\sigma_p^2 \quad (5)$$

can be established as an optimal fit (highest probability and lowest number of independent variables; $r^2 = 0.892$; $n = 13$; $p < 0.025$). The correlation function containing parameters for the position 2 only (i.e., terms σ_p and σ_p^2) is also somewhat significant ($r^2 = 0.425$; $n = 13$; $p < 0.1$). On the other hand, the data cannot be fitted by the function composed exclusively from linear terms of all constants ($r^2 = 0.266$; $n = 13$; $p < 0.1$). It should be mentioned in passing that a very tight correlation exists if the resonance constant $\mathfrak{R}$ of Swain and Lupton for substituents in position 1 and the 2 terms with σ_p and σ_p^2 (position 2) are considered. The $\mathfrak{R}$-constant has apparently no physical meaning in this case of aliphatic substitution and can only be explained by an accidental correlation with some other, more physically relevant constants. This is here indeed the case: for our group of N-terminal substituents and also with 14 other similar N-substituents listed in the table by Hansch et al.[16], there is such a correlation between $\mathfrak{R}$, π and R_m. This should be taken as a warning against a formal use of statistical analysis in structure-activity studies of similar kind.

Conclusion. The following conclusions can be drawn: 1. The physical properties of substituents on the N-terminal of the oxytocin molecule have only a weak influence on its binding to the uterus receptor. 2. The resonance effects of p-substituting groups on 2-tyrosine show a considerable effect on the binding. There is a certain optimal level of the resonance effect which can be expressed in terms of the Hammett constant as $\sigma_p(\text{optimal}) = -0.152 \pm 0.014$ (arithmetic mean of values obtained by means of various correlation models±SD). 3. There are no apparent correlations to the lipophilicity of position 2; the lipophilic contribution of position 1 is rather dubious. This lack of relation was already reported for position 2 in oxytocins[19] and more recently found also for position 1 in another, not dissimilar case, namely that of the uterotonic effect of N^{α}-substituted angiotensins[20]. 4. Although position 1 exerts rather weak effects upon binding, the contributions of the 2 positions to the receptor binding are approximately additive and the concept of Free and Wilson is, to a large extent, valid in this case.

These results are based on a data set which is far from being optimally suited to these aims. Frequently, pure intuition is considered by many peptide chemists to be the best strategy in investigations of structure-activity relationships. The obvious consequence of such an intuitive approach is that semiquantitative methods like those considered here, which have been used for decades with other biologically active substances, cannot be fully applied. The outcome of the laborious and time consuming syntheses is consequently in many cases of rather modest importance for structure-activity studies. For a successful study of this type, one would prefer a large number of permutations of even a limited number of substituents at each position investigated, rather than many isolated substitutions, as was the case here.

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Effect of methyl ester of aristolic acid from *Aristolochia indica* Linn. on fertility of female mice

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Summary. Methyl ester of aristolic acid, a pure compound isolated from the roots of *Aristolochia indica* (Linn.), was found to exert 100% abortifacient activity at a single oral dose of 60 mg/kg b.wt when administered on 6th or 7th day of pregnancy; 20 and 25% abortifacient effect were observed at the same dose on day 10 and 12, respectively.

The crude petroleum ether extract of the roots of *Aristolochia indica* Linn. was reported to have 100% interceptive activity[2] in mice when fed on day 6 or 7 of pregnancy at a single oral dose of 100 mg/kg b.wt. Aristolic acid, a pure compound isolated from the chloroform extract of the same plant material has also been found to exert abortifacient activity in 100% treated mice at the dose level of 60 mg/kg b.wt similarly administered[3]. We now report the effect of methyl ester of aristolic acid, $C_{18}H_{14}O_5$ (mol.wt 310), m.p. 172 °C encountered in the petroleum ether and benzene extracts of *Aristolochia indica* roots and also obtained by treatment of diazomethane on aristolic acid[4], in mice.

Materials and methods. Colony bred Swiss albino fertile female mice weighing 24–25 g were caged with proved males in the ratio of 2:1 at a controlled room temperature (24–25 °C). The day the copulation plug was in place was marked as day 1 of pregnancy. The test compound was pasted with gum acacia and suspended in water for oral administration. It was fed orally with the help of a gastric canula at a single dose level of 60 mg/kg b.wt on day 6 or 7 of pregnancy. After establishing the antifertility activity of the compound, it was administered at successive lower dose levels of 50, 40 and 30 mg/kg b.wt for elucidation of dose response relationship. Laparotomy was performed on days