# CARBA ANALOGS OF NEUROHYPOPHYSIAL HORMONES

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Pioneering syntheses[1] of the neurohypophysial hormones oxytocin and vasopressin carried out in the 1950s led to the possibility of their study by synthesis of suitably modified analogs. About 600 have been prepared so far. The preparation of analogs with a modified disulfide bridge (for examples see References 2 to 4 and references given in Table 2; for a survey see Reference 5) which is one of the very important structural features of these compounds, led both to a modification of the views on the mechanism of action of neurohypophysial hormones,[3] and to the attainment of compounds with distinctly increased biological activities.[6,7] For the purification of synthetic compounds we used a number of methods (gels, ion-exchange and partition chromatography, free-flow electrophoresis, and counter-current distribution). We have recently substituted reversed-phase high performance liquid chromatography (RP-HPLC) for these methods. Special stress is laid on the so-called lyophilizable mobile phase (0.1% trifluoroacetic acid (TFA), 0.1 *M* triethylammonium trifluoroacetate at pH 4 to 5, 0.05 *M* ammonium acetate at pH 6 to 7.5, 0.05 *M* triethylammonium carbonate at pH 7 to 8.5). When nonvolatile buffer components are used, the eluates from preparative chromatography should be desalted[8] on Sep-Pak® $C_{18}$ cartridges. (Note: their capacity for deaminooxytocin is at least 3 mg per cartridge.)

Substitution of sulfur by a methylene group increases the lipophilic character[9] of the compound and when chromatographed on a reversed phase column should exhibit a corresponding increase in the capacity factor value in comparison with the nonmodified substance. However, in the case of carba-analogs of oxytocin we observed the opposite effect,[10] which was consistent with the behavior of some cyclic dipeptides[10] which differed in sulfur content. When comparing simple compounds, such as protected methionine and norleucine, it may be observed, however, that they behave as expected (Table 1), i.e., the norleucine derivative has a longer retention time. Therefore, we compared the behavior of some protected and free peptides which were intermediary products of the synthesis of carba-analogs of oxytocin, differing in the content and position of the sulfur atom. A protected tetrapeptide (IIIc) — in agreement with the assumption of the increased lipophilicity of compounds with a $CH_2$ group replacing the sulfur atom — was eluted later than the peptides containing sulfur. In the case of pentapeptides (IVa-c) the differences in retention times are already minimal or zero. In free heptapeptides (Va-c) the compound without sulfur was eluted between the two substances containing sulfur, while in the case of protected octapeptides (VIa-c) the order was analogous. On passing to cyclic peptides the situation changes dramatically; compounds containing a $CH_2$ group instead of sulfur (X-XI) are eluted sooner (see Table 2) and the retention times of the analogs with different positions for sulfur differ considerably. The same is true of the analogs containing an α-amino group (VII, VIII), a contracted cyclic structure (XIII, XIV), or a modified amino acid in position 2 (XLIII, XLIV). In analogs of vasopressin (XIX-XXIII) a distinct dependence of the k value on the pH of the mobile phase is evident (see Figure 1). This dependence is of greater difference in the carba-6-analog (XXI) than in the carba-1-analog (XX) which is similar to a compound with a preserved disulfide bridge (XIX). In contrast to this the carba-6-analog containing D-arginine (XXIII) displays a pH-dependence similar to that of the disulfide analog (XXII).

A complete omission of the disulfide bridge, connected with the elimination of the cyclic structure[11] (in compound XVII) leads to a decrease of the retention time, while the substitution of cystine by two *S*-methylcysteines (XVIII) leads to an increased retention; both these facts may be predicted by estimating the lipophilicity change in the analog formed.

The position of the substitution for sulfur in the disulfide bridge can alter the effect of

**Table 1**
**REVERSED-PHASE CHROMATOGRAPHIC K' VALUES OF SYNTHETIC INTERMEDIATES OF SOME CARBA-ANALOGS**

| Compound | Structure[a] | $k'^{b,d}_{S}$ | $k'^{c,d}_{SO}$ | Ref. |
|---|---|---|---|---|
| Ia | Boc-Met-OH | 1.96 | 1.01 | 28 |
| Ib | Boc-Nle-OH | 3.16 | | 28 |
| IIa | Boc-Cys($C_3H_6COOMe$)-OH | 2.12 | 1.11 | 29 |
| IIb | Boc-Hcy($C_2H_4COOMe$)-OH | 1.92 | 1.09 | 23 |
| IIIa | Z-Cys($C_3H_6COOMe$)-Pro-Leu-Gly-$NH_2$ | 3.06 | 1.91 | 3 |
| IIIb | Z-Hcy($C_2H_4COOMe$)-Pro-Leu-Gly-$NH_2$ | 2.81 | 1.67 | 4 |
| IIIc | Z-Asu(OMe)-Pro-Leu-Gly-$NH_2$ | 3.30 | | 4 |
| IVa | Nps-Asn-Cys($C_3H_6COOMe$)-Pro-Leu-Gly-$NH_2$ | 1.99 | | 3 |
| IVb | Nps-Asn-Hcy($C_2H_4COOMe$)-Pro-Leu-Gly-$NH_2$ | 1.80 | | 4 |
| IVc | Nps-Asn-Asu(OMe)-Pro-Leu-Gly-$NH_2$ | 1.97 | | 4 |
| Va | H-Ile-Gln-Asn-Cys($C_3H_6COOH$)-Pro-Leu-Gly-$NH_2$ | 2.88[e] | 2.22[e] | 3 |
| Vb | H-Ile-Gln-Asn-Hcy($C_2H_4COOH$)-Pro-Leu-Gly-$NH_2$ | 2.53[e] | 1.81[e] | 4 |
| Vc | H-Ile-Gln-Asn-Asu-Pro-Leu-Gly-$NH_2$ | 2.61[e] | | 4 |
| VIa | Boc-Tyr($Bu^t$)-Ile-Gln-Asn-Cys($C_3H_6COOH$)-Pro-Leu-Gly-$NH_2$ | 5.84 | 4.98 | 3 |
| VIb | Boc-Tyr($Bu^t$)-Ile-Gln-Asn-Hcy($C_2H_4COOH$)-Pro-Leu-Gly-$NH_2$ | 5.61 | 4.39 | 4 |
| VIc | Boc-Tyr($Bu^t$)-Ile-Gln-Asn-Asu-Pro-Leu-Gly-$NH_2$ | 5.62 | | 4 |

[a] Asu, α-aminosuberic acid, Hcy, homocysteine, Nle, norleucine.
[b] Values for sulfide form.
[c] Values for corresponding sulfoxide.
[d] Separon SI-C-18 (25 × 0.4 cm), 0.05% TFA-methanol (30:70).
[e] Same as for d but (50:50).

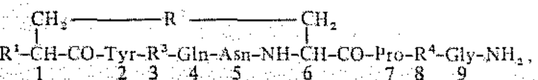
substitution on retention time. Substitution of sulfur in position 1 has a substantially lower effect on the retention characteristics than the same substitution in position 6 (see Table 2). This again confirms the nonequivalence of the two sulfur atoms which had previously been established with the biological activities of carba-analogs[7-12] and from the study of their CD spectra.[13] On the basis of these spectra an interaction of the sulfur atom in position 6 with the aromatic ring of tyrosine (i.e., in carba-1-analogs) was demonstrated and therefore it may be assumed that this sulfur atom is less accessible for other interactions. On the other hand, the elimination of the interaction between sulfur and the aromatic ring by a carba-substitution of this atom may lead to an increase in the freedom of movement of the tyrosine side chain, which is one of the most important structural elements responsible for the interaction of the peptide with the stationary phase. Thus this substitution can lead to a more dramatic change in retention than the substitution of sulfur in position 1, which is oriented to the "other side" of the molecule and is less likely to interact with the hydrophobic stationary phase. The original assumption[10] that the shielding of sulfur at position 1 inside the molecule was shown to be false by a kinetic study of the oxidation of carba-analogs,[14] where the deamino-6-carba-oxytocin (XI) was oxidized substantially more rapidly than the 1-carba-analog (X). Inspection of a model[15,16] (considering an interaction of the aromatic ring with the sulfur in the 6-position) also shows that access for the relatively small and hydrophilic molecules of the oxidant to the sulfur atom is easier in position 1. The slow rate of oxidation of sulfur at position 6 could be due to the sulfur-aromatic ring interaction or to the steric hindrance to the access of the oxidant by the side chain of the amino acid in position 2. We studied the kinetics of the oxidation of analogs containing the sterically demanding *tert*-leucine in position 2. Again a more rapid oxidation of the compound containing sulfur in position 1 was observed,[17] even though the observed difference was slightly smaller than for substances containing tyrosine.[14] Thus, it may be said that the shielding

Table 2
REVERSED-PHASE CHROMATOGRAPHIC K′ VALUES OF SOME CARBA-ANALOGS

| Compound | Structure[a] | k′[b] | Mobile phase[c] | k′[b] | Mobile phase[c] | Ref. |
|---|---|---|---|---|---|---|
| VII | OT | 4.18 | A 50 | 2.00 | B 50 | 1 |
| VIII | $C^{1}OT$ | 3.97 (3.19) | A 50 | 1.83 (1.56 + 1.60) | B 50 | 30 |
| IX | dOT | 4.98 | A 50 | 3.22 | B 50 | 31 |
| X | $dC^{1}OT$ | 4.32 (3.20) | A 50 | 2.[illegible] (1.9[illegible] + 2.22) | B 50 | 3 |
| XI | $dC^{6}OT$ | 11.7 (8.56) | A 40 | 2.00 (1.55) | B 50 | 4 |
| XII | $dC^{1}C^{6}OT$ | 3.35 | A 50 | 1.98 | B 50 | 4 |
| XIII | $dCH_2^{1-6}OT$ | 2.63 | A 50 | 1.62 | B 50 | 32 |
| XIV | $dS^{1-6}OT$ | 4.17 (2.83) | A 50 | 2.54 (1.97) | B 50 | 32 |
| XV | $dCH_2SCH_2^{1-6}OT$ | 4.26 (2.65) | A 50 | 2.57 (1.51) | B 50 | 33 |
| XVI | $dC^{1}OT\text{-}SO_2$ | | | 4.72 | B 50 | 12 |
| XVII | $Ala^{1}$, $Ala^{6}OT$ | 2.70 | A 50 | | | 11 |
| XVIII | $Cys(Me)^{1}$, $Cys(Me)^{6}OT$ | 6.28 (3.09) | A 50 | | | 11 |
| XIX | $Arg^{8}dVP$ | 2.70 | A 50 | 8.70 | C 40 | 34 |
| XX | $Arg^{8}dC^{1}VP$ | 2.34 (2.14) | A 50 | 6.48 (4.84) | C 40 | 35 |
| XXI | $Arg^{8}dC^{6}VP$ | 3.13 (2.73) | A 50 | 5.76 (4.61) | C 40 | 36 |
| XXII | $D\text{-}Arg^{8}dVP$ | 2.98 | A 50 | 10.8 | C 40 | 37 |
| XXIII | $D\text{-}Arg^{8}dC^{6}VP$ | 2.82 (2.63) | A 50 | 9.84 (7.98) | C 40 | 36 |
| XXIV | $Ile^{2}\text{-}dC^{1}OT$ | 9.81 (7.64) | A 50 | 6.43 (3.78 + 6.15) | B 50 | 38 |
| XXV | $Tle^{2}dC^{1}OT$ | 3.78 (2.83) | A 55 | 4.69 (3.19 + 3.63) | E 55 | 17 |
| XXVI | $Phe^{2}dC^{1}OT$ | 13.6 (10.3) | A 50 | 8.72 (5.52 + 8.11) | B 50 | 38 |
| XXVII | $Tyr(Me)^{2}dC^{1}OT$ | 13.1 (8.29) | A 50 | 8.42 (5.62 + 6.74) | B 50 | 38 |
| XXVIII | $Phe(F)^{2}dC^{1}OT$ | 3.04 (2.00 + 2.55) | E 65 | 7.22 (4.11 + 6.16) | B 50 | 18 |
| XXIX | $Tyr(J_2)^{2}dC^{1}OT$ | 5.43 (3.62 + 4.64) | A 60 | | | 29 |
| XXX | $Glu^{4}dC^{1}OT$ | 1.59 (1.20) | A 63 | 2.33 (1.53 + 1.75) | B 50 | 39 |
| XXXI | $Tyr(J_2)^{2}$,$Glu^{4}dC^{1}OT$ | 4.52 (3.27 + 3.98) | A 63 | | | 29 |
| XXXII | $Ile^{2}dC^{6}OT$ | 9.33 (7.96) | A 50 | 6.18 ([illegible]) | B 50 | 40 |
| XXXIII | $Tle^{2}dC^{6}OT$ | 3.66 (2.90) | A 55 | 4.59 (3.65) | E 55 | 17 |
| XXXIV | $Phe(NH_2)^{2}dC^{6}OT$ | 1.76 | A 55 | 2.23 (1.65) | D 55 | 23 |

## Table 2 (continued)
## REVERSED-PHASE CHROMATOGRAPHIC K′ VALUES OF SOME CARBA-ANALOGS

| Compound | Structure[a] | k[b] | Mobile phase[c] | k[b] | Mobile phase[c] | Ref. |
|---|---|---|---|---|---|---|
| XXXV | $Phe(NO_2)^2dC^6OT$ | 4.68 (3.72) | A 55 | 6.41 (5.25) | D 55 | 23 |
| XXXVI | $Tyr(Me)^2dC^6OT$ | 5.01 (4.23) | A 55 | 6.81 (5.56) | D 55 | 40 |
| XXXVII | $Phe^2dC^6OT$ | 5.26 (4.39) | A 55 | 7.27 (5.77) | D 55 | 23 |
| XXXVIII | $Phe(NMe_2)^2dC^6OT$ | 2.92 (2.21) | A 55 | 13.4 (11.2) | D 55 | 23 |
| XXXIX | $Tyr(Et)^2dC^6OT$ | 8.01 (6.64) | A 55 | 11.4 (9.11) | D 55 | 23 |
| XL | $Phe(Me)^2dC^6OT$ | 8.79 (7.36) | A 55 | 12.7 (9.78) | D 55 | 23 |
| XLI | $Phe(Cl)^2dC^6OT$ | 5.05 (4.19) | A 60 | 15.0 (11.7) | D 55 | 23 |
| XLII | $Phe(NHZ)^2dC^6OT$ | 14.2 (12.0) | A 55 | 22.9 (18.6) | D 55 | 23 |
| XLIII | $Phe(Et)^2dC^6OT$ | 4.69 (4.02) | A 65 | 23.4 (18.0) | D 55 | 23 |
| XLIV | $Phe(Et)^2dOT$ | 5.73 | A 65 | 27.7 | D 55 | 29 |
| XLV | $D\text{-}Tyr^2dC^6OT$ | 11.2 (5.30) | A 40 | 13.6 (6.52) | C 40 | 29 |
| XLVI | $D\text{-}Phe(Cl)^2dC^6OT$ | 6.98 (5.91) | A 60 | 19.8 (14.8) | D 55 | 29 |

[a]



OT denotes oxytocin ($R^1$ = $NH_2$, $R^2$ = –S–S–, $R^3$ = Ile, $R^4$ = Leu), VP denotes vasopressin ($R^1$ = $NH_2$, $R^2$ = –S–S–, $R^3$ = Phe, $R^4$ = Arg or Lys), d means deamino ($R^1$ = H), $C^1$ means 1-carba ($R^2$ = $-CH_2-S-$), $C^6$ means 6-carba ($R^2$ = $-S-CH_2$), $C^1C^6$ means di-carba ($R^2$ = $-CH_2-CH_2-$), $CH_2^{1,6}$, $S^{1,6}$, $CH_2SCH_2^{1,6}$ means that $R^2$ = $-CH_2-$, –S–, $-CH_2SCH_2-$.

[b] Value for corresponding sulfoxide is given in parentheses.

[c] A, 0.1% TFA B, 0.08 *M* triethylammonium trifluoroacetate buffer of pH 4.5; C, 0.05 *M* ammonium acetate buffer at pH 7.0, D, 0.01 *M* triethylammonium borate buffer at pH 8.1, E, 0.01 *M* sodium phosphate buffer at pH 4.4, the number indicates the percentage of methanol in the mixture.

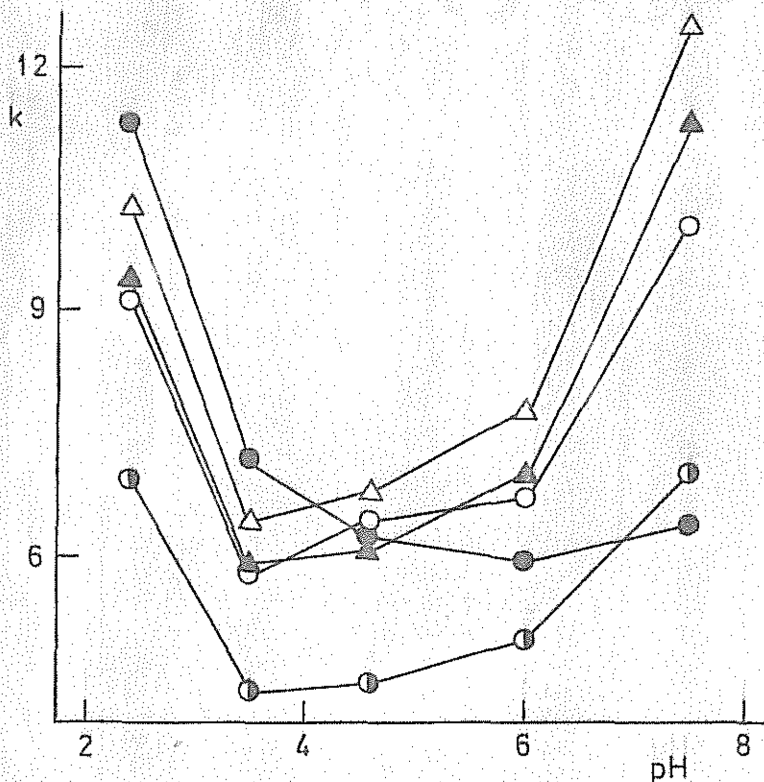



FIGURE 1. Dependence of k values of vasopressin analogs on pH of the mobile phase. ○, [8-Arginine]deamino-vasopressin; ◑ - [8-Arginine]deamino-1-carba-vasopressin; ● - [8-arginine]deamino-6-carba-vasopressin; △ - [8-D-arginine]deamino-vasopressin; ▲ - [8-D-arginine]deamino-6-carba-vasopressin. Conditions: Separon SI-C-18 (25 × 0.4 cm), 0.05 *M* phosphate buffer of different pH – methanol (60:40), flow 1.5 mℓ/min.

effect is at least partly steric in nature. We also compared the oxidation rate of carba-analogs of vasopressin;[14] in these compounds the oxidation is considerably faster than in oxytocin derivatives. No important difference in oxidation rate, dependent on the position of sulfur atom, was observed in this case. For comparison the kinetics of the oxidation of substance P was also studied. Substance P is oxidized several orders of magnitude more rapidly. All kinetic experiments were carried out separately and they were checked using HPLC. To compare the oxidation rates, competition experiments may be used, where both substrates (present in equal concentrations) are oxidized simultaneously (see Figure 2).

With carba-1-analogs of oxytocin a separation of diastereoisomeric sulfoxides may be achieved[10,14] in depending on their structure and the mobile phase used. The most different elution characteristics were observed with sulfoxides of [2-*p*-fluorophenylalanine]deamino-1-carba-oxytocin,[18] which could be separated even with a methanol-water mixture. Proof that the substances were indeed diastereoisomeric sulfoxides was by reducing the isolated sulfoxides, which led to identical sulfides. When these were oxidized, identical mixtures of sulfoxides were formed in both cases.[18] Oxidation with sodium periodate and reduction with hydrogen bromide and acetone (monitored by HPLC) became our routine laboratory method for checking the degree of oxidation of sulfur in synthetic substances.[10,14] Examples of the behavior of sulfides and sulfoxides in reversed-phase chromatography may be found in Table

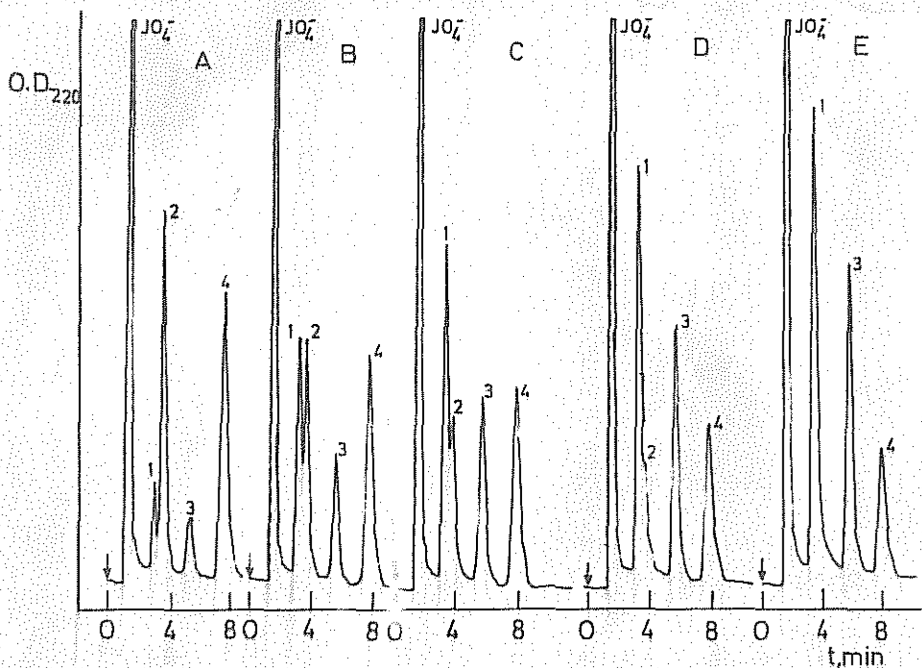



FIGURE 2. Oxidation of a mixture of [8-arginine]deamino-1-carba-vasopressin (2) and deamino-1-carba-oxytocin (4) to corresponding sulfoxides (2 → 1, 4 → 3) by sodium periodate. Conditions: Separon SI-C-18 (25 × 0.4 cm), 0.1% TFA – methanol (55:45), flow 1.5 mℓ/min. (A) Immediately after periodate addition; (B) after 10 min; (C) after 20 min; (D) after 32 min; (E) after 43 min.

1 and 2. The separation of diastereoisomeric sulfoxides could not be achieved with an acidic or neutral mobile phase unless an amine had been added. Triethylammonium trifluoroacetate buffer at pH 4 to 4.5 was the best mobile phase. A phosphate buffer with a similar pH may also be used.[14] Diastereoisomeric sulfoxides of carba-vasopressins were separated with a buffer of pH 6.4. However, in none of the mobile phases tested were we able to achieve the separation of sulfoxides of 6-carba-analogs of oxytocin. The explanation is again based on the assumption that interaction of the sulfur atom in position 6 with the aromatic ring is prevented by oxidation and the configuration of the sulfoxide leads to a different orientation of the tyrosine side chain. (We tried to use this fact — under assumption of the validity of the oxytocin model considered[15,16] — for the prediction of the absolute configuration of individual stereoisomers of sulfoxides.[14,18]) By contrast the oxidation of sulfur in position 1 does not significantly influence the conformation of the tyrosine. However, similar behavior is not limited to analogs containing an aromatic ring in position 2. The analogs containing a *tert*-leucine in this position (XXV, XXXIII) differ only slightly in their capacity factors;[17] nevertheless, the 1-carba-analog affords on oxidation a separable mixture of diastereoisomers, while the 6-carba-analog does not.

Using preparative HPLC we separated the diastereoisomers of some sulfoxides and determined their biological activity.[14,18] The diastereoisomers of 1-carba-analog mostly differ in activity by as much as several orders of magnitude.[14,18]

In the case of deamino-1-carba-oxytocin, the activity of the less active isomer approaches that of the sulfone (XVI) rather than of the other diastereoisomer. In sulfoxides of 6-carba-analogs the activity is decreased relatively little in comparison with the sulfides, which may

be explained by the fact that the steric arrangement of the elements important for eliciting receptor response (which are[19] the tyrosine, and asparagine side chains) is not significantly disturbed. In the case of carba-analogs of vasopressin it may be seen[14] again that the oxidation of a 1-carba-analog leads to a much greater decrease in activity and that the difference of k values between diastereoisomers is also larger.

We also investigated the degradation rate of *tert*-butyl-sulfonium salts of deamino-1-carba and 6-carba-oxytocin.[20] The salt derived from 6-carba-oxytocin is much more stable which again shows that the model[16] considering the interaction of the side chain of the amino acid residue in position 2 with the sulfur atom in position 6 is correct, since the model would predict the destabilization of the sulfonium salt of the 1-carba-analog.

If a single structural feature (i.e., substituent) is changed in the whole molecule, then the chromatographic behavior of the substance formed should be predictable on the basis of a change in lipophilicity,[21] unless a change in the conformation of the whole molecule takes place.[22] We prepared[23] a number of analogs of deamino-6-carba-oxytocin differing merely by substitution of the para position of the aromatic nucleus of the amino acid in position 2 (XXXIV-XLIII). Retention characteristics of these substances were well correlated[24] with the $\pi$-values of corresponding substituents and thus it is probable that these analogs do not differ in conformation even though they differ substantially in their biological activities.[23]

Substitution of the aromatic nucleus of tyrosine by two iodine atoms in positions 3 and 5 led to a very pronounced increase in the elution time of the analog (X, XXX, and XXIX, XXXI). Using HPLC we observed that when the iodination was carried out in alkaline medium,[25] both for deamino-1-carba-oxytocin (X) and for the analog (XXX), oxidation of sulfur did not take place. The sulfoxides formed by oxidation with periodate were easily separable into individual diastereoisomers.

We also used HPLC to follow the enzymatic cleavage of the carba-analogs. The effect of the post-proline cleaving enzyme (EC 3.4.21.26) was better observed with deamino-1-carba-oxytocin, than with oxytocin as this analog did not inactivate the enzyme; (oxytocin evidently blocks its free SH group.) The action of chymotrypsin on analogs which did not contain an aromatic amino acid in the position 2, gave analogs without glycine amide at the C-terminus of the molecule. These analogs are interesting for their activities in affecting the CNS. For a preparative purification of these compounds it is advantageous to use volatile buffers at neutral pH. At this pH the peptide with a free carboxyl group is eluted earlier than the starting analog (when using an acid mobile phase it is eluted later).

Using RP-HPLC separation of diastereoisomeric peptides may be achieved.[26,27] We made use of this fact in the synthesis of carba-analogs containing unnatural amino acids in position 2. In Figure 3 an elution profile[41] is shown for the preparative chromatography of the crude reaction mixture after cyclization of [2-D,L-*p*-chlorophenylalanine]deamino-6-carba-oxytocin. A similar profile was also observed in the preparation of the analog containing a disulfide bridge, or an amino group, or some other modification in position 1. When the analog XLV containing D-tyrosine in position 2 was prepared, it was essential to eliminate any traces of the substance containing this amino acid of L-configuration (XI). On comparison with a standard it was found, however, that both analogs differ from one another only very slightly ($\alpha$ = 1.05), while their sulfoxides give an $\alpha$ = 1.47. The analog was therefore purified by conversion to the sulfoxide which was purified by preparative chromatography, reduced and rechromatographed. The whole operation was carried out on a 10-mg scale within one working day.

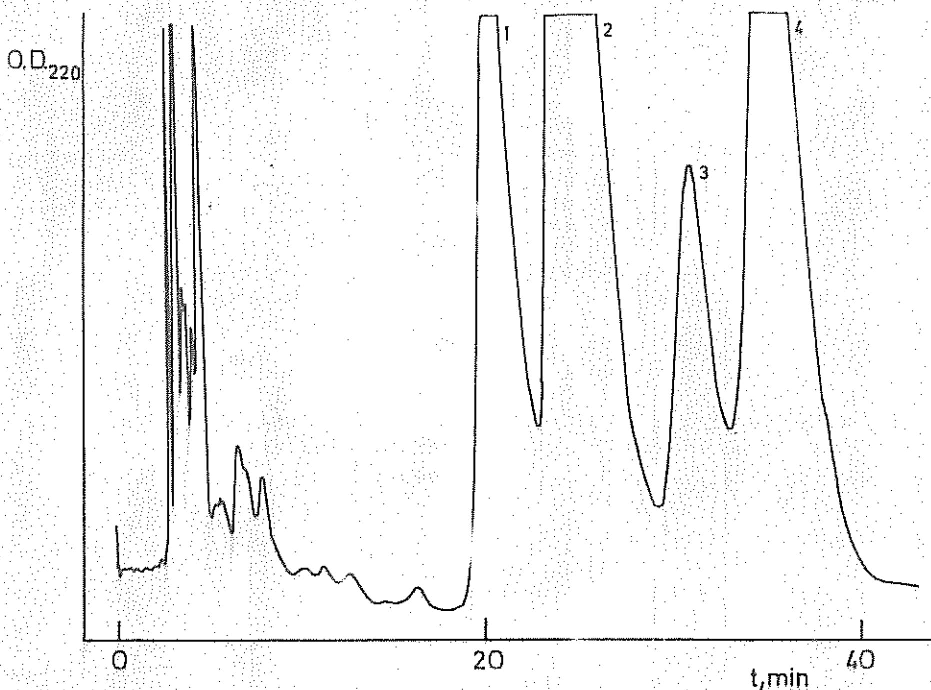



FIGURE 3. Preparative chromatography of the crude mixture after cyclization of carba-analog. [2-L-*p*-chlorophenylalanine]deamino-6-carba-oxytocin (2), its sulfoxide (1), [2-D-*p*-chlorophenylalanine]deamino-6-carba-oxytocin (4) and its sulfoxide (3). Conditions: Partisil® ODS-2 (50 × 0.9 cm), 0.05% TFA – methanol (45:55), flow 5 mℓ/min, load 28 mg.

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