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CYCLIC MELANOTROPINS. PART VI*. REVERSE PHASE HPLC STUDIES

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ABSTRACT

The chromatographic behavior of 17 cyclic [Cys^4, Cys^{10}]-α-melanocyte stimulating hormone (α-MSH, α-melanotropin) analogs were studied on two reversed phase columns (Altech and Vydac) using several mobile phases. It was observed that analogs which contain a D-amino acid were always eluted earlier than the corresponding L-amino acid-containing analogues. Substitution of penicillamine for cysteine in the 4 position led to a more lipophilic compound as expected, but when penicillamine was substituted in the 10 position, apparently a less lipophilic compound resulted. These observations can be interpreted as a result of a conformational change in the molecule caused by the particular substitution. Furthermore, decreasing the size of the intramolecular disulfide ring led to a decrease in lipophilicity (i.e. retention time). The carba modification of the disulfide bridge had a similar effect as previously seen in oxytocin and vasopressin, that is, a decrease in retention time.

* For Part V see (16).

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INTRODUCTION

High performance liquid chromatography (HPLC) is one of the most powerful methods for the separation and isolation of organic compounds. It has been applied to almost all types of biologically active molecules, of which peptides comprise an important class. Problems in the chromatography of peptides and proteins have been discussed in monographs (1,2) and in many papers devoted to the subject.

Reversed phase high pressure liquid chromatography (RP-HPLC) allows the separation of compounds which differ only slightly in their covalent structure, three-dimensional structure, or other physical-chemical properties. Minor structural modifications often can have unpredictable effects on the retention times of peptides, especially if the modification causes a change in the conformation of the molecule which results in changes of its overall lipophilicity. If, within a series of minimally modified peptide analogs, the introduction of a more lipophilic moiety, such as replacement of H by CH_3 leads to increased retention in RP-HPLC, then (3) the conformations of the analogs are probably similiar. However, if substitution with a more lipophilic substituent leads to decreased retention, then a change in conformation would be predicted.

Several good examples of this behavior have been found in studies of diastereomeric peptides (4-10). For example, two peptides which are identical except for the stereochemistry of one amino acid, should have essentially the same lipophilicity, since

the lipophilicity of the side chains is not altered by a change in stereochemistry. However, in the case of neurohypophysial hormones almost all reported peptides containing one amino acid residue of the D-configuration have longer retention times than the all L-amino acid analogs (5-8, 11). This implied that this substitution lead to a conformational change in the peptide to one of greater lipophilicity (6). It is well known that the conformation of many peptides are strongly solvent dependent (12) and that the conformation can change with the pH or the ionic strength of the solution. Therefore, any comprehensive chromatographic study which desires to examine and understand the behavior of a peptide should utilize a variety of mobile phases as well as stationary phases (13). In this paper we have studied a series of cyclic [$\overline{Cys^4, Cys^{10}}$]-α-MSH analogs which differ only slightly in their overall structure with the aim of understanding how these structural changes are reflected in their chromatographical behavior and have interpreted the results of our investigation in terms of the parameters discussed above.

EXPERIMENTAL

The cyclic melanotropins studied were synthesized at the University of Arizona utilizing the general procedures developed for these peptides in our laboratory (14-17). High performance liquid chromatography was performed on 25 x 0.46 cm I.D. columns, packed with C18 reversed phase material (16 μm (Vydac) or 5 μm (Altech)) using a Spectra Physics SP-8700 liquid chromatograph

equipped with an SP-8400 continuously variable wavelength UV detector (Spectra Physics, Santa Clara, CA, U.S.A.). We used a flow rate of 1 ml/min and 220 nm UV detection to monitor the peptides. Solvents used as mobile phases were of UV grade and were filtered before use through Millipore filters.

RESULTS AND DISCUSSION

We have examined the chromatographic behavior of several cyclic α-MSH analogs which were prepared in our laboratory as part of our examination of conformation-biological activity relationships of this hormone. The cyclic structure of α-MSH (Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2) was designed based upon the "pseudoisosteric" replacement of methionine-4 and glycine-10 in the native hormone with a disulfide bridge formed via two cysteine residues substituted in these two positions (14). The reverse turn structure imposed by this substitution, encompasses the residues -His(6)-Phe(7)-Arg(8)-Trp(9)-, and is believed to provide the preferred conformation required for biological activity (14-18). Structures of the compounds studied are shown in Fig. 1.

The results of the chromatographic studies utilizing two different C18 column packings (Vydac and Altech) and several mobile phases, are summarized in Table 1 and Fig. 2. It can be clearly seen that the Vydac column has a much greater selectivity and a wider range of retention times for structurally similar compounds. Conversely, the Altech column is more efficient

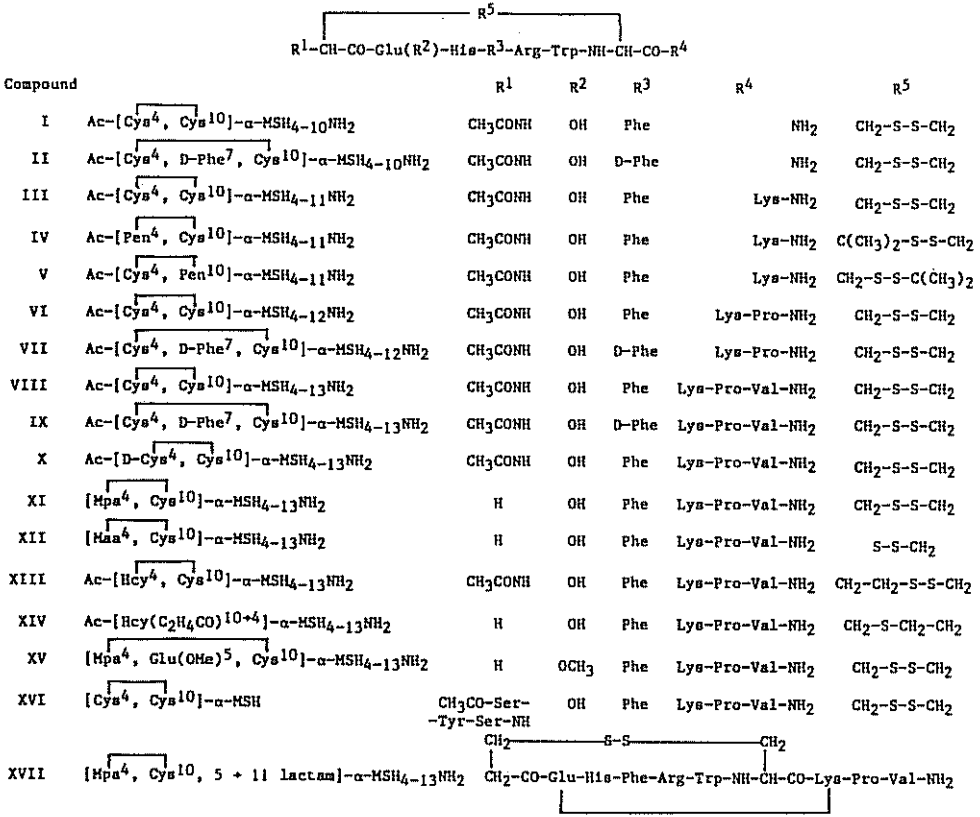

Figure 1: Structures of α-MSH cyclic analogs investigated.

TABLE I

Values of k' under various conditions of chromatography.

Compound	Column and Conditions[a]			
	A	B	C	D
I	2.80	2.69	2.59	6.41
II	2.07	1.55	1.48	2.90
III	1.73	1.64	1.54	2.72
IV	2.25	2.38	3.07	5.46
V	----	1.30	1.41	2.19
VI	1.89	1.62	1.78	2.97
VII	1.41	1.00	0.90	1.45
VIII	2.75	2.72	4.24	7.14
IX	1.79	1.32	1.41	2.29
X	2.64	2.52	3.02	6.03
XI	3.19	3.25	3.81	8.58
XII	2.93	2.79	3.43	6.65
XIII	3.53	4.83	9.62	18.1
XIV	2.36	1.93	2.25	4.24
XV	5.27	4.96	5.86	14.9
XVI	----	----	4.34	----
XVII	6.60	7.41	9.55	~28.1[b]

a) A: Altech C-18 column, 30% of acetonitrile in 0.5% trifluoroacetic acid.
B: Vydac C-18 column, 21% of acetonitrile in 0.1% trifluoroacetic acid.
C: Vydac C-18 column, 37% of methanol in 0.25 M triethylammonium phosphate pH 2.2.
D: Vydac C-18 column, 15% of acetonitrile in 0.25 M triethylammonium phosphate pH 2.2.

b) Estimated from elution with 20% of acetonitrile.

(i.e. has more theoretical plates) than the Vydac column, which is partially due to the different mesh size of the silica gel beads (5 μm vs. 16 μm, respectively). These two columns also show different responses to the trifluoroacetic acid (TFA) buffer concentration. On both columns increasing the TFA concentration

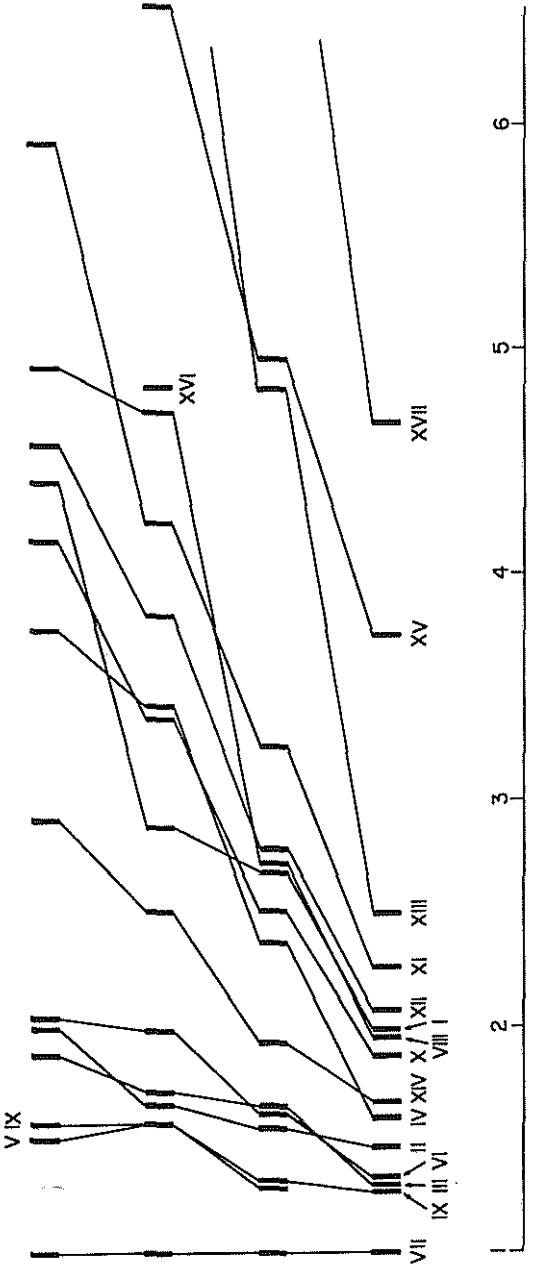

Figure 2: Comparison of relative retention of cyclic α-MSH analogs. Values are calculated relative to k′ of compound VII. Columns and conditions A-D are the same as in Table II.

TABLE II

Calculated and Observed Relative Retention Times of Compounds I, III, VI, and VIII.

Compound	Relative Retention Time[a]					
	Calculated[b]		Observed[c]			
	1	2	A	B	C	D
I	0.0	0.0	0.0	0.0	0.0	0.0
III	-1.9	-3.0	-0.7	-1.06	-1.2	-2.5
VI	2.5	0.1	-0.66	-1.08	-0.9	-2.3
VIII	8.4	4.7	-0.05	0.03	1.8	0.5

(a) Numbers are only relative and have no absolute value. A negative number implies that the compound is eluted earlier than compound I, which has a value of 0.

(b) These are calculated for different increment values obtained with various mobile phases (1- 0.1M $NaClO_4$, 0.1% H_3PO_4, gradient of acetonitrile; 2- 0.1 M NaH_2PO_4, 0.1% H_3PO_4, gradient of acetonitrile). The reported values are a sum of increments (20, 21) for additional amino acids, when sequence 4-10 is taken as zero.

(c) Conditions A-D are described in Table I. Values are calculated according to formula: $k'_A/k'_{VII} - k'_I/k'_{VII}$ where k'_A is the value of k' for the given compound (I, III, VI, VII, VIII).

from 0.10% to 0.15% leads to an increase in column efficiency (980 to 1060 theoretical plates in the case of the Altech column, and 504 to 1424 in the case of the Vydac column - in all cases efficiency calculations are based on compound XI). Interestingly, the elution times decrease (about 3.1 times) on the Altech column and increase (about 1.5 times) on the Vydac column as the TFA content increases. The best overall separation of the

compounds studied are achieved in a 0.25 M triethylammonium phosphate (pH 2.2) buffer with acetonitrile as the organic modifier (19). Buffers containing 0.10% trifluoroacetic acid can be used for preparative purifications, but the column is less efficient with the TFA buffer than it is with the phosphate buffer. In addition, prolonged use of 0.10% TFA can lead to a loss of the bonded alkyl chains on the stationary phase (20).

Separations based on the length of the peptide chain are obtained with methanol as the organic modifier in the mobile phase. Cyclic compounds containing the 4-11 (III) and 4-12 (VI) α-MSH sequences are difficult to separate in the trifluoroacetic acid-containing buffer (α = 1.01) as are the 4-10 (I) and 4-13 (VIII) α-MSH sequences (α = 1.01). This problem is overcome with a methanolic triethylammonium phosphate buffer system, which provides excellent separation (α = 1.16 and 1.64, respectively) for these pairs of compounds. Substituting methanol for acetonitrile with the same buffer, the separation is worse (α = 1.09 and 1.11, respectively) (See Fig. 3).

Interestingly, prediction of the elution order for cyclic melanotropins (Table II), based on the amino acid sequence (20, 21), was: Ac-[$\overline{Cys^4, Cys^{10}}$]-α-$MSH_{4-11}$-$NH_2$, Ac-[$\overline{Cys^4, Cys^{10}}$]-α-$MSH_{4-10}$-$NH_2$, Ac-[$\overline{Cys^4, Cys^{10}}$]-α-$MSH_{4-12}$-$NH_2$, and Ac-[$\overline{Cys^4, Cys^{10}}$]-α-$MSH_{4-13}$-$NH_2$. The observed order, utilizing the best conditions for separation (conditions C) was: Ac-[$\overline{Cys^4, Cys^{10}}$]-α-$MSH_{4-11}$-$NH_2$, Ac-[$\overline{Cys^4, Cys^{10}}$]-α-$MSH_{4-12}$-$NH_2$, Ac-[$\overline{Cys^4,}$

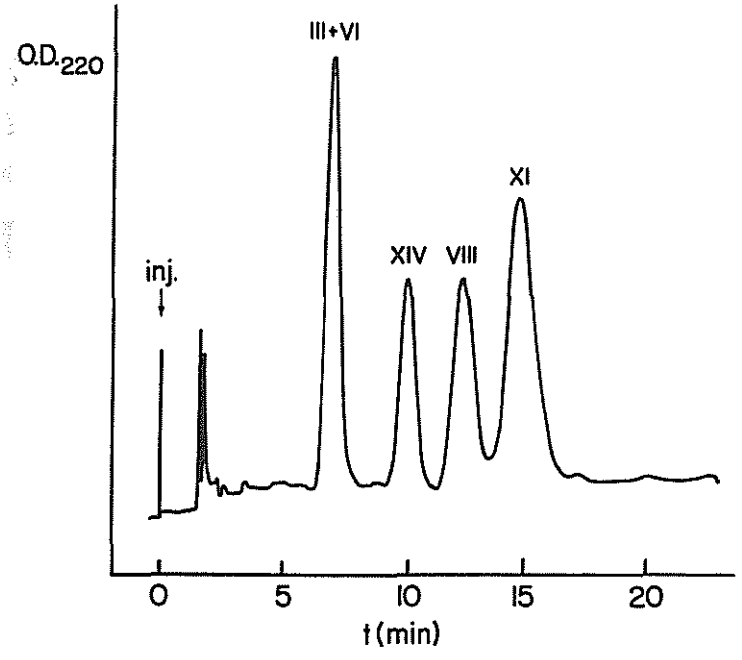

Figure 3: Separation of cyclic analogs III, VI, VIII, XI and XVI. Vydac C-18 column, 2 ml/min, acetonitrile (17% v/v)- 0.1% trifluoroacetic acid (83% v/v).

$\overline{Cys^{10}}$]-α-MSH$_{4-10}$-NH$_2$, and Ac-[$\overline{Cys^{4}, Cys^{10}}$]-α-MSH$_{4-13}$-NH$_2$. These results suggested that the effect of proline on the retention time in these reversed phase systems was less than expected. The smaller influence of proline on the retention times of the 4-12 fragment (VI) could be due to its carboxyl-terminal position, since its effect was different from compounds which contain proline inside the peptide chain. A cyclic analog containing the 1-13 sequence of α-MSH, [$\overline{Cys^{4}, Cys^{10}}$]-α-MSH (XVI), was eluted very close to the 4-13 cyclic analog (VIII), but due to a lack of

material it was only studied in one mobile phase. The relative retention time of the 1-13 sequence versus that of the 4-13 analog was in agreement with the prediction based on amino acid sequence, when the contribution of tyrosine was practically negated by the two serine residues (21, 22).

Interestingly, compounds containing D-phenylalanine in position 7 (D-Phe-7) exhibit very prolonged *in vitro* (23) and *in vivo* (18) biological activities. All D-Phe-7 containing compounds were eluted earlier than the corresponding L-Phe-7 diastereomeric analogs under all the experimental conditions utilized. Interestingly, changing from methanol to acetonitrile led to a very pronounced increase in the difference of retention times. For example, L-Phe-7 and D-Phe-7 analogs with the 4-10 sequence (I and II) had α values corresponding to 1.75 with methanol and 2.21 with acetonitrile, respectively. This difference was less pronounced for analogs containing the 4-13 (VIII and IX) sequence (α = 3.00 for methanol and α = 3.12 for acetonitrile, respectively). On the contrary, a decrease in the difference of retention times is observed in going from a mobile phase containing methanol to one containing acetonitrile when the stereochemistry of cysteine is changed (L-Cys to D-Cys) in the 4 position (VIII and X) (α = 1.40 with methanol and α = 1.18 with acetonitrile, respectively).

Substitution of penicillamine for cysteine in the 4 or 10 position, (compounds IV and V, respectively), should lead to an analog of greater lipophilicity. Indeed, an increase in the

retention time of the compound with penicillamine in position 4 was observed, but with penicillamine in position 10 a decrease in retention time was observed. This suggested that the Pen-10 compound exhibits a change in the overall conformation of the molecule resulting in the inaccessibility of that residue for interaction with the stationary phase.

Substitution of an N-terminal acetylamino group for hydrogen leads to an increase of elution times in buffers with acetonitrile as the organic modifier (compounds VIII and XI, see Fig. 3). In the case of methanol as the organic modifier, the elution order of compounds VIII and XI are reversed. A decrease in the size of the ring by deletion of one methylene group leads to a decrease in retention times (compounds XI and XII) in all systems used. Increasing the size of the ring (by adding a methylene group) leads to an increase in retention times (compounds VIII and XIII), which is especially dramatic on the Vydac column using the triethylammonium phosphate buffer. In both cases the change in the retention time could be predicted from the structural change, that is, both changes occur as a result of the changes in lipophilicity of the substitution. Increasing the size of the ring results in a larger than expected increase in retention time, and apparently, this is probably due to a larger than expected increase in lipophilicity. The longer retention time of the homocysteine-containing compound (XIII) can be explained by greater freedom of the backbone in this analog, resulting from

the increase in the intramolecular ring size. Carba modification of the disulfide bridge in the melanotropins (compound XIV), which were previously studied in the case of oxytocin and vasopressin analogs (24, 25), leads to compounds with shorter retention times upon comparison to the disulfide bridged molecule (XI, see Fig. 3). Similar results were observed in the oxytocin and vasopressin analogs. Methylation of the free carboxylic group of glutamic acid in position 5 (compound XV) increases the retention times as compared to the unesterified peptide. Introduction of a second intramolecular bridge (compound XVIII), caused a similar increase in its relative retention time.

From the data presented, it is clear that even very small structural differences can express themselves by a change in chromatographic behavior. Nonetheless, if careful comparative studies are made, conclusions about conformational similarities can be extracted from the structure-lipophilicity relationships and their expression in the retention time observed for analogs on reverse phase HPLC systems. Further verification of our tentative conclusions will require careful physio-chemical studies of the described compounds. Such studies are in progress in our laboratory.

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