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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY STUDIES OF α-MSH FRAGMENTS

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SUMMARY

α-Melanotropin (α-MSH) is a linear tridecapeptide (Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2), that is primarily known for its ability to stimulate melanosome dispersion within integumental melanocytes (F. J. H. Tilders, D. F. Swaab and T. B. van Wimersma Greidanus (Editors), *Frontiers of Hormone Research*, Vol. 4, Karger, Basel, 1977; J. Ramachandran, S. W. Farmer, S. Liles and C. H. Li, *Biochim. Biophys. Acta*, 428 (1976) 347). In our efforts to understand the relationships of structure and conformation to the biological activities of α-MSH, we have prepared a series of diastereoisomeric analogues based on the highly potent analogue Ac-[Nle^4, D-Phe^7]-α-MSH_{4-11}-NH_2 (T. K. Sawyer, V. J. Hruby, B. C. Wilkes, M. T. Draelos, M. E. Hadley and J. Bergsneider, *J. Med. Chem.*, 25 (1982) 1022). These analogues differed only in the amino acid substituted in the seven position, which was thought to be a critical residue for the biological activity of α-MSH. The chromatographic behavior of these analogues was examined on a C_{18} Vydac (16-μm) reversed-phase column with five different mobile phases. The selectivity (α) for the analogues was compared in 0.10% trifluoroacetic acid (TFA), 0.10% heptafluorobutyric acid (HFBA) and 0.25 *M* triethylammonium phosphate (TEAP) using either acetonitrile or methanol as the organic modifier. With only one exception all analogues substituted with a D-amino acid in the seven position were eluted prior to their L-amino acid counterparts. As expected due to the greater ionic strength, the TEAP buffer allowed the greatest selectivity for the separation of these α-MSH analogues, but it was surprising to find that the TFA buffer had a greater influence on selectivity than the HFBA buffer with either organic modifier. The probable mechanism of retention for the α-MSH analogues in perfluoroalkanoic buffers was also investigated. In addition, the relationship between the retention time and the hydrophobicity of the seven position substitution was examined. Although the data were somewhat limited, a lack of correlation between the hydrophobicity of the seven position residue and retention time was observed.

INTRODUCTION

Recently high-performance liquid chromatography (HPLC) has become one

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of the most powerful methods for the separation and purification of polyfunctional organic compounds. Its application to peptide and protein chemistry has met with many problems, which have been reviewed by many authors[1,2].

Reversed-phase high-performance liquid chromatography (RP-HPLC) has been utilized for the separation of compounds which differ only slightly in their overall lipophilicity. In many cases, modifications of a molecule will have unpredictable results on the retention times in RP-HPLC. However, if in a series of minimally modified analogues, replacement of a particular moiety by a more lipophilic moiety leads to an increase in the retention times observed on RP-HPLC, then the conformations of the molecules are probably similar[3,4]. Likewise, the converse is true; that is, if substitution of a more lipophilic moiety leads to a decrease in retention on RP-HPLC, then the conformations of the analogues are probably different. Changing the stereochemistry of one amino acid in a peptide will not change the lipophilicity of that particular residue and should not change the overall lipophilicity of the peptide unless there is a change in conformation.

Several examples of this behavior have been found in the study of diastereomeric peptides[4–12]. For example, in neurohypophysial hormones, substitution of a D-amino acid for an L-amino acid results in analogues with longer retention times on an RP-HPLC column which indicates that this substitution leads to a conformation of greater lipophilicity[3–6,13]. Likewise, a similar situation has been reported for cyclic α-melanotropin (α-MSH) analogues of varying chain lengths, except that in this case substitution of a D-amino acid in the seventh or fourth position led to shorter retention times on RP-HPLC[12]. These results indicated that the D-amino acid containing α-MSH analogue is of lower lipophilicity than the corresponding all L-amino acid containing peptide.

Examination of the differences between the retention of a series of analogues in a variety of buffer systems can give some insight into the mechanism of retention. Theoretical mechanisms for retention have been based upon the interactions between the sample, solvent and the stationary phase, including both adsorption and electrostatic relationships. These two concepts have been incorporated into two different models, the ion-pairing[14] and dynamic ion-exchange model[15], respectively. More recently, a third model, the ion-interaction model[16], has been developed which stresses the importance of both adsorption and electrostatic forces acting in cooperation to bring about the observed retention.

In the present study, we undertook the analysis of the chromatographic behavior of a series of fourteen linear α-MSH fragments which only differ in the amino acid substituted in the seven position. We used three different aqueous buffer systems along with either acetonitrile or methanol as the organic modifier. Perfluoroalkanoic acid buffers were chosen based upon prior experience in this laboratory[12] and reports in the literature on their utility as possible ion-pairing reagents[17–21]. The concentration of the organic acid (0.10%) has been reported to be optimal for the separation of a variety of peptides[21]. A phosphate buffer, which was reported to provide high resolution and high recovery of various peptides[22] has also been examined. The retention mechanism in this buffer was reported to be a function of the ionic strength and pH, and not necessarily equated to its ion-pairing ability. In particular, we examined the behavior of the analogues in the following buffers: 0.10% trifluoroacetic acid (TFA) pH 2.0; 0.10% heptafluorobutyric acid (HFBA) pH 2.0; and 0.25 *M*

triethylammonium phosphate (TEAP) pH 2.2. The results of this study are interpreted in terms of the above discussion.

EXPERIMENTAL

The linear melanotropins used in this study were synthesized via solid phase methodology on a *p*-methylbenzhydrylamine resin using methods previously reported[23]. The peptides were cleaved from the resin with anhydrous liquid hydrofluoric acid, and then purified by gel filtration, ion-exchange chromatography or RP-HPLC as required. The purified fragments were chromatographed at room temperature on a Vydac column No. 830822 (25 × 0.46 cm I.D.) packed with C_{18} reversed-phase (16-μm) material. We utilized a Spectra-Physics SP-8700 liquid chromatograph equipped with an SP-8400 variable-wavelength UV detector (Spectra-Physics, Santa Clara, CA, U.S.A.) for all of the studies reported here. The elution of compounds was detected at 214 nm with a flow-rate between 1.5 and 2.5 ml/min with the mobile phases indicated in Table I. All solvents were UV grade and filtered through Millipore filters prior to use. Distilled water obtained from our department's distilled deionized water supply was further purified by passing through the Norganic filtration system. All solvents were thoroughly degassed prior to use and were continually purged with helium during the experiment. All isocratic mixtures were prepared prior to use and the Vydac column was thoroughly equilibrated before starting the experiment.

RESULTS AND DISCUSSION

Fourteen synthetic analogues of Ac-α-MSH_{4-11}-NH_2 were studied on a Vydac C_{18} (16-μm) reversed-phase column with five different solvents systems (Table I). All of the analogues contained a Nle residue in the 4 position and one of the following amino acids in the 7 position: Gly, D- or L-Ala, D- or L-Tyr, D- or L-Pgl (phenylglycine), D- or L-Tic (tetrahydroisoquinoline carboxylic acid), D- or L-Phe, L-Tla (thienylalanine) and D- or L-*p*-nitrophenylalanine. These solvent systems utilized the following buffers: 0.10% TFA, pH 2.0; 0.10% HFBA, pH 2.0; and 0.25 *M* TEAP, pH 2.2 with varying concentrations of acetonitrile or methanol as the organic modifier (see Table I for the specific solvent systems used).

Where possible, RP-HPLC conditions were modified in such a manner that the compound with the shortest retention time (*i.e.*, smallest capacity factor k') has approximately the same k' in all systems to allow a reasonable basis for comparison. For the TEAP–acetonitrile system we were not able to utilize this approach due to the great selectivity of this system (Table I, Fig. 1). In this system in order to monitor most of the analogues in an isocratic system, it was necessary to choose conditions such that the fastest eluting analogues had shorter retentions (k') than in the other systems analyzed.

In examining the retention times for the various analogues, it was observed that in virtually all cases fragments substituted with a D-amino acid in the seven position were eluted prior to their L-amino acid counterparts (Figs. 1 and 2). This was also seen with the cyclic melanotropin analogues[12]. An exception to this generalization was seen when one compared the behavior of L-*p*-NO_2-Phe^7 with D-*p*-NO_2-Phe^7 in which the D-amino acid containing analogues elutes after the L-con-

TABLE I

VALUES OF k' WITH VARIOUS MOBILE PHASES*

*Compound***		*Conditions****				
No.	*R*	*A*	*B*	*C*	*D*	*E*
I	Gly	1.38	1.15	0.59	1.53	1.52
II	D-Ala	1.61	1.30	0.82	1.53	1.94
III	Ala	1.77	1.30	0.91	1.67	2.12
IV	D-Tyr	2.69	1.60	1.82	1.80	3.47
V	Tyr	4.15	1.70	4.00	2.67	5.29
VI	D-Pgl	3.62	1.85	2.54	2.07	4.00
VII	Pgl	7.69	2.30	5.82	2.93	9.24
VIII	D-Tic	—§	—§	3.91	2.80	7.35
IX	Tic	6.69	2.15	5.64	3.00	10.18
X	D-Phe	8.85	2.55	7.91	3.40	12.41
XI	Phe	15.77	3.25	15.18	5.07	25.24
XII	Tla	11.15	2.75	9.82	3.93	15.47
XIII	D-*p*-NO_2	—§	4.45	—§	4.53	—§
XIV	*p*-NO_2	16.54	3.50	—§	4.40	20.4

* All compounds were chromatographed on a C_{18} Vydac (25 × 0.46 cm I.D.) 16-μm reversed-phase column.

** Compounds are Ac-[Nle^4, R^7]-α-MSH_{4-11}-NH_2, where R is an amino acid of the L-configuration unless noted otherwise.

*** A = TFA–acetonitrile (80:20), 2.5 ml/min; B = HFBA–acetonitrile (70:30), 1.5 ml/min; C = TEAP–acetonitrile (86:14), 1.5 ml/min; D = HFBA–methanol (50:50), 2.0 ml/min; E = TFA–methanol (68:32), 2.0 ml/min.

§ Not determined.

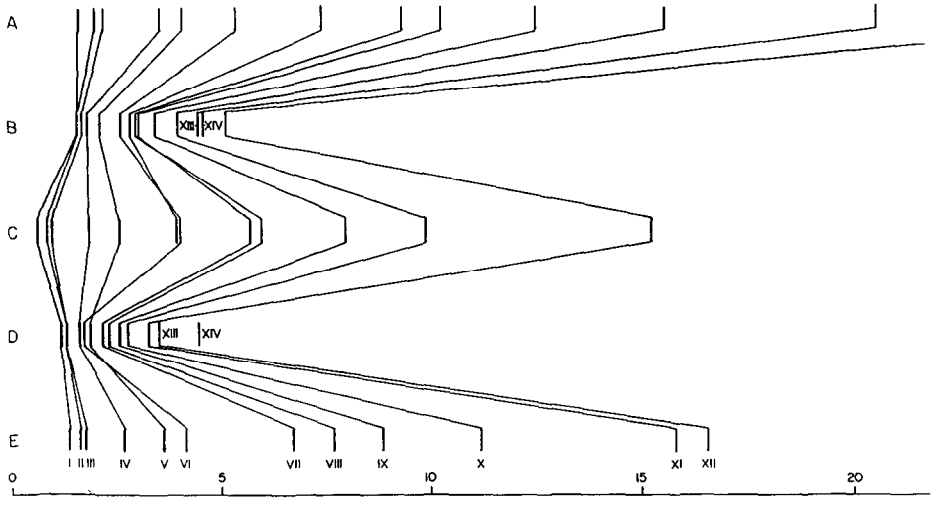

Fig. 1. A plot of the k' (abscissa) of the α-MSH fragments studied *versus* the various mobile phases (ordinate) utilized on a C_{18} Vydac (25.0 × 0.46 cm I.D., 16-μm) column. The compounds and mobile phases employed are defined in Table I.

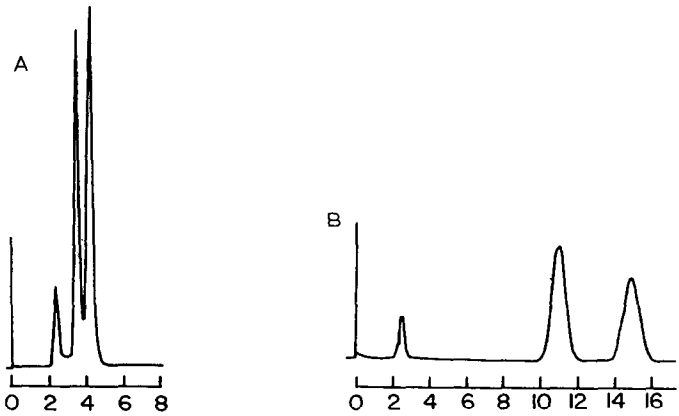

Fig. 2. An illustration of the separation of a variety of position 7 substituted linear melanotropin fragments in solvent system C [0.25 *M* TEAP–acetonitrile (86:14); 1.5 ml/min]. A, The peaks from left to right correspond to the solvent front, Ac-[Nle^4, Gly^7]-α-MSH$_{4-11}$-NH$_2$ and Ac-[Nle^4, Ala^7]-α-MSH$_{4-11}$-NH$_2$. B, The peaks from left to right correspond to the solvent front, Ac-[Nle^4, D-Tic^7]-α-MSH$_{4-11}$-NH$_2$ and Ac-[Nle^4, Tic^7]-α-MSH$_{4-11}$-NH$_2$.

taining diastereoisomer. Unfortunately, due to the extremely long retention times of these analogues with the TFA or the TEAP buffers (Table I, Fig. 1) it was only possible to make this comparison in the HFBA buffer with either acetonitrile or methanol as the organic modifier.

Since the retention times on RP-HPLC are dependent upon the overall lipophilicity of a molecule, it should be possible to make some inferences about the lipophilicity differences between minimally modified compounds based upon retention times (k'). If the lipophilicity of these α-MSH analogues (based upon retention times) are compared (Table I), it can be seen that in all cases the substitution of a D-amino acid in the seven position of Ac-α-MSH$_{4-11}$-NH$_2$ leads to a less lipophilic analogue, except for the substitution of a D-*p*-NO$_2$-Phe, which leads to a compound of higher overall lipophilicity. Since the lipophilicity of an individual amino acid is the same for either the L or D optical isomers, it is apparent that the change in lipophilicity of the peptide analogue must be due to a change in conformation and/or overall topology of the peptide.

The correct selection of a buffer system for any series of compounds in RP-HPLC, requires a careful analysis of the probable mechanism of retention. In many cases, it is possible to make some inferences into this mechanism based upon the physical properties of both the mobile phase and the compounds to be analyzed. In this series of buffers, the pH was either 2.0 or 2.2 which implied that in these particular α-MSH fragments, glutamic acid, histidine, arginine and lysine are all fully protonated. Accordingly, this could provide an environment in which lipophilic anions (trifluoroacetate or heptafluorobutyrate) could form ion pairs with the three basic amino acids (His, Arg and Lys). Indeed, it had been reported that buffers containing small amounts (0.10%) of perfluoroalkanoic acids of varying chain length can have dramatic effects on both retention and selectivity[17,18]. In particular, it was shown that both retention and selectivity increased with increasing the chain length of the ion-

pairing reagent, being especially dramatic for peptides with a large number of basic amino acids[17].

If the method of separation in these α-MSH analogues is ion-pairing, the retention on RP-HPLC should be governed by the number of basic groups on the ionized solute and the lipophilicity of the counter-ion. Since in all the analogues studied the number of basic amino acids remains constant and only the lipophilicity of the counter-ion is altered, the retention time should be governed by this change. Indeed, as predicted, we observed greater retention times for the analogues in the HFBA buffer (more lipophilic counter-ion) than in the TFA buffer with either acetonitrile or methanol as the organic modifier (Table I, Fig. 1). These results are not consistent with the dynamic ion-exchange model[15] in which the lipophilic ion is absorbed onto the stationary phase and the retention is controlled simply by the number and strength of the ionic groups of the solute. Neither of these properties were altered by changing from a TFA buffer to a HFBA buffer. Even though the results are more consistent with an ion-pairing mechanism, it is not inconceivable that both mechanisms are in operation and we are, in fact, experiencing an ion-interaction mechanism[16].

Furthermore, it has been reported that selectivity (α) can be increased by increasing the chain length of the ion-pairing reagent[17]. Interestingly for the analogues reported here, the *selectivity* seen for the individual α-MSH fragments was significantly greater in the TFA system (Table II, Fig. 3) with either methanol or acetonitrile as the organic modifier. In comparing the selecitivity differences between acetonitrile and methanol with the TFA buffer it is obvious that methanol allows a slightly, but consistently, greater selectivity. Interestingly, however, the selectivity of the HFBA buffer is much less dependent on the organic modifier (Table II, Fig. 3). Furthermore, based upon selectivity, it is apparent that TEAP buffer provides the best system for the separation of linear 4–11 fragments of α-MSH (Table II and Figs. 1 and 3), which had also been shown to be the best system for the separation of cyclic analogues of

TABLE II

SELECTIVITY, α, FOR VARIOUS MOBILE PHASES

*Compound**		*Conditions**				
No.	*R*	*A*	*B*	*C*	*D*	*E*
III	Ala	1.28	1.13	1.54	1.09	1.39
V	Tyr	2.90	1.47	6.78	1.74	3.48
VII	Pgl	5.57	2.00	9.86	1.92	6.08
IX	Tic	4.85	1.86	9.56	1.96	6.70
XI	Phe	11.43	2.82	25.73	3.31	16.61
II	D-Ala	1.17	1.13	1.38	1.00	1.28
IV	D-Tyr	1.95	1.39	3.08	1.17	2.28
VI	D-Pgl	2.62	1.61	4.30	1.35	2.63
VIII	D-Tic	–**	–**	6.63	1.83	4.83
X	D-Phe	6.41	2.22	13.41	2.22	8.16

* As defined in Table I.

** Not determined.

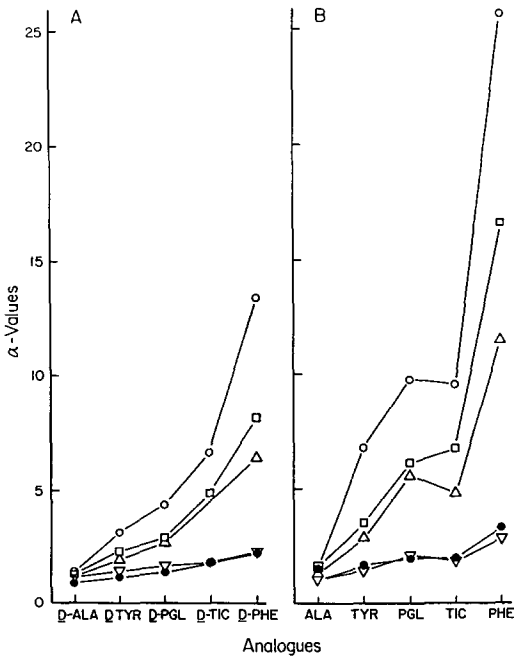

Fig. 3. The selectivity {α = k' (analogue)/k' (Ac-[Nle^4, Gly^7]-α-MSH$_{4-11}$-NH$_2$)} between Ac-[Nle^4, Gly^7]-α-MSH$_{4-11}$-NH$_2$ and various analogues substituted with a D-amino acid (A) or an L-amino acid (B) in the seventh position in various solvent systems; A, △; B, ▽; C, ○; D, ●; E, □.

α-MSH (ref. 15). This could be expected due to the higher ionic strength of this buffer when compared to the TFA and HFBA systems. However, TEAP is not as convenient a buffer for preparative purification as TFA or HFBA, since salts of the latter two buffers can be removed by lyophylization, while the former buffer is not as volatile.

In these peptide fragments we have only altered position 7 of the α-MSH analogues and thus it should be possible to correlate the retention times of Phe^7, Tyr^7, and *p*-NO$_2$-Phe^7 with their respective hydrophobicities (π values)[3]. The hydropho-

TABLE III

COMPARISON OF k' *VERSUS* π VALUES* FOR SELECTED ANALOGUES WITH PERFLUORINATED CARBOXYLIC ACID BUFFERS

*Compound***		π	*Conditions***			
No.	*R*		*A*	*B*	*D*	*E*
V	Tyr	−0.7	4.15 (0.62)***	1.70 (0.23)	2.67 (0.43)	5.29 (0.72)
XI	Phe	0.0	15.77 (1.20)	3.25 (0.51)	5.07 (0.71)	25.24 (1.40)
XIV	*p*-NO$_2$	−0.3	16.54 (1.22)	3.50 (0.54)	4.40 (0.64)	20.47 (1.31)

* π = Hydrophobicity constant for a given single substituent on a benzene ring[16]; values are uncorrected.

** Conditions as defined in Table I.

*** Values in parentheses correspond to log k'.

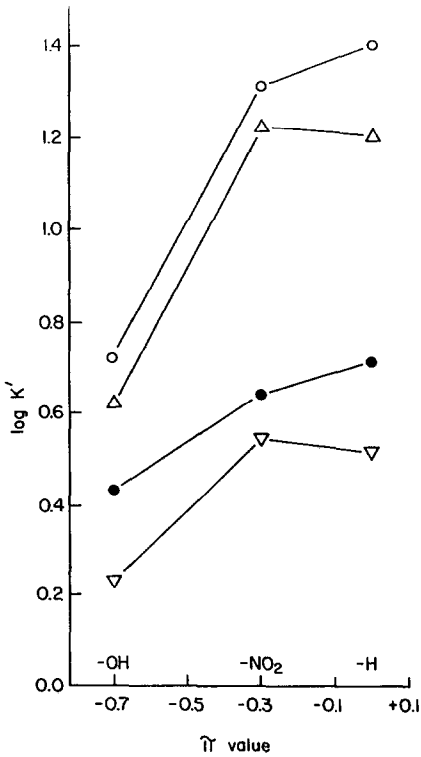

Fig. 4. A plot of the correlation of the hydrophobicity of various *para* substituents on a phenyl ring (π) *versus* log k' observed on the reversed phase, for α-MSH analogues containing appropriately substituted phenylalanine derivatives in the seven position of the hormone analogue in four different mobile phases: A, △; B, ▽; D, ●; E, ○.

bicity value for an individual *para*-substituted aromatic amino acid is based upon the corresponding hydrophobicity of an appropriately substituted phenyl ring as compared to benzene[24]. This was previously shown to be a valid argument for a series of oxytocin analogues[3]. Surprisingly, however, this is not the case for α-MSH, since there is not a linear correlation (Table III and Fig. 4) between log k' and the π values for these analogues. In particular, the L- or D-*p*-NO_2 compound does not correlate well at all. Interestingly, when methanol is used as the organic modifier as opposed to acetonitrile the correlation between the hydrophobicity values (π) and retention is somewhat better.

In conclusion, we have shown that in most cases the substitution of a D-amino acid for the corresponding L-amino acid in the seven position in both linear and cyclic analogues of Ac-α-MSH_{4-11}-NH_2 leads to analogues of less lipophilicity based upon comparison of their retention times. Since the overall lipophilicity of the individual amino acid was not changed, the observed changes in retention time is probably due to a conformational or a topological effect. In analyzing the ability of differing mobile phases to separate linear melanotropic fragments with only slight structural differences, the favored buffer system is the TEAP buffer, followed by the

TFA buffer (based on α values). It appears from the data that the method of retention in perfluoroalkanoic acid buffers is most consistent with an ion-pairing mechanism. In addition, the organic modifier of choice for use with TFA appears to be methanol. Thus for analytical work, the best system is TEAP–acetonitrile, but for preparative work in which it is desirable not to have to desalt the peptide after RP-HPLC purification, TFA–methanol provides the best results.

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