SYNTHESIS AND BIOLOGICAL ACTIVITY OF NEW METALLOCENIC ANGIOTENSIN II ANALOGS

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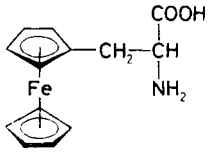
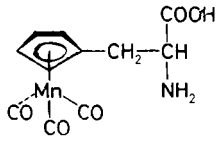
Dedicated to the memory of Dr Karel Bláha.

Analogs of angiotensin II in which phenylalanine in position 8 was replaced with cymantrenylalanine or with its triphenylphosphine photosubstitution product were synthesized by the solid-phase method. On rabbit aorta strips, these peptides were found to be pure antagonists of angiotensin II. Their relative affinities are higher than most other analogs substituted in position 8 with bulky amino-acids.

Replacement of one or more amino-acids in a chain with a non-coded counterpart is probably the most straightforward means by which a peptide chemist can modulate physical, chemical and biological properties of a peptide[1]. An important contribution of K. Bláha, in this field was the design of various amino-acids in which bulkiness or hydrophobicity operate at an extreme degree[2-6]. While in the case of aliphatic side-chains, steric and hydrophobic interactions play the major role, π-donor properties can be a third factor of great importance with aromatic amino-acids. Due to their unique structure, organometallic π-complexes offer the possibility of increasing the thickness and hydrophobicity of the aromatic system while retaining its charge-transfer capacity. Incorporation of ferrocenylalanine (Fer) *I* in different peptides was reported almost simultaneously by three groups among which K. Bláha's[7-9].

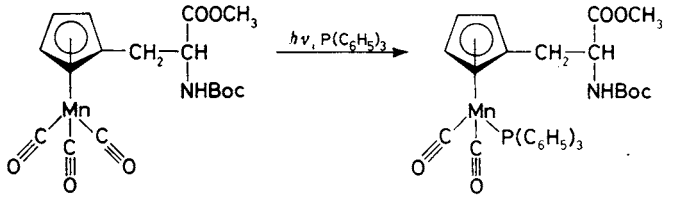




An other organometallic amino-acid, cymantrenylalanine[10] (Cym) *II* proved to be stable enough to withstand chemical treatments used during classical solid-phase

peptide synthesis. In addition to its use as a highly hydrophobic aromatic residue (log *D* 1·2 as determined by partition of its N^{α}-acetyl carboxamide derivative in octanol–water[11]) cymantrenylalanine is a photosensitive structure where, under appropriate irradiation conditions a carbonyl group can be replaced with various ligands. This substitution allows to modify easily the bulkiness and hydrophobicity around the metal atom; an other interest being its possible use in photoaffinity labeling. We describe in this paper our attempts to use these possibilities in the case of [Sar[1]]angiotensin II (Sar-Arg-Val-Tyr-Ile-His-Pro-Phe) in which phenylalanine in position 8 was replaced by cymantrenylalanine.

Cymantrenylalanine was prepared via the azlactone route[10] and converted to its N^{α}-Boc protected derivative as previously described[12]. No attempt was made at this step to resolve both enantiomers as it was expected that, due to the bulkiness of the side chain, the resulting angiotensin diastereoisomers could be easily separated. Photosubstitution of a carbonyl ligand according to Scheme 1 was carried out by irradiation (about 280 nm) of N^{α}-Boc-Cym methyl ester *III* in the presence of a 1·5 fold excess of triphenylphosphine.



III *IV*

SCHEME 1

N^{α}-Boc-Cym *III* or its triphenylphosphine derivative N^{α}-Boc-Cym-$P(C_6H_5)_3$ *IV* were reacted as their cesium salts with a chloromethylated polystyrene resin. [Sar[1], Cym[8]]AT II and [Sar[1], Cym-$P(C_6H_5)_3^8$]AT II were synthesized acording to classical Boc-TFA solid phase method[13]. Syntheses were performed under nitrogen and exposure to day-light was avoided. After treatment with hydrogen fluoride due to their limited solubility, peptides were first purified by chromatography on Sephadex LH20. Diastereoisomers were separated by preparative reversed-phase HPLC and named 1 or 2 corresponding respectively to the faster and slower eluting peaks.

Amino-acid compositions were determined after acid hydrolysis except for metallocenic residues which were destroyed under these conditions. The presence of the intact organometallic moieties could be monitored by the typical pattern in IR spectroscopy of the strong carbonyl stretching bands[14] between 1 800 and 2 100 cm^{-1}. Moreover, the relative intensities of these bands reflect the local symmetry around the metal atom in each complex, as can be seen in Fig. 1.

RESULTS AND DISCUSSION

Biological activities were tested in vitro on rabbit aorta strips. The results are presented in Table I and compared with other AT II analogs substituted in posi-

TABLE I

Biological activity of position 8 modified [Sar^1]AT II analogs

Peptide	Relative affinity[a]	
	L	D
[Sar^1, Ala^8]AT II (ref.[16])	100	—
[Sar^1, Phe^8]AT II (ref.[17])	agonist	38
[Sar^1, Leu^8]AT II (ref.[18])	74	23
[Sar^1, Phe(Br$_5$)^8]AT II[b] (ref.[19])	17	15
[Sar^1, Car^8]AT II[b] (ref.[20])	0·15	—
[Sar^1, Ada^8]AT II[b] (ref.[21])	5·5	—
[Sar^1, Fer^8]AT II[c] (ref.[22])	1·3	1·3
[Sar^1, Cym^8]AT II[c]	8·5	5·6
[Sar^1, Cym P(C$_6$H$_5$)$_3$^{8}]AT II[c]	12	7·8

[a] The relative affinities are expressed in % of the ID_{50} values versus the standard antagonist saralasin ([Sar^1, Ala^8]AT II) with an ID_{50} value of 2·57 nmol. ID_{50} is the dose of antagonist that reduces the response of a double dose of agonist to that of a single dose; [b] Phe(Br$_5$) is (2,3,4,5,6-pentabromophenylalanine, Car is carboranylalanine, Ada is adamantylalanine; [c] D and L configurations have been attributed on the basis of their relative elution times in reversed-phase HPLC. This is done in analogy to the HPLC separation of diastereoisomeric pairs containing Phe, Phe(Br)$_5$ and Leu, where the D-diastereoisomer always emerges after the L-diastereoisomer.

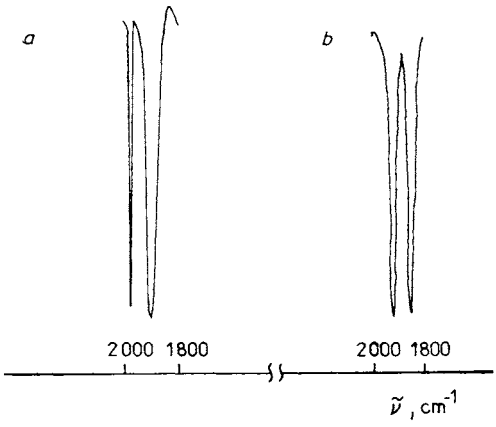


FIG. 1

Carbonyl stretching bands between 1 800 and 2 100 cm^{-1} for *a* [Sar^1, Cym^8]AT II and *b* [Sar^1, Cym P(C$_6$H$_5$)$_3$^{8}]AT II

tion 8. In each case, the slower eluting compound was less active than the faster one, a result which is consistent with the fact that in this series, the D-isomer containing diastereoisomer is always the slower eluting.

Both [Sar^1, Cym^8]AT II and [Sar^1, Cym-P$(C_6H_5)_3^8$]AT II exhibit antagonist activities. Relative affinities were less important that those of [Sar^1, Ala^8]AT II and [Sar^1, Leu^8]AT II. However, except for [Sar^1, Phe$(Br_5)^8$]AT II, they remained higher than most other analogs substituted in position 8 with bulky amino-acids. Of particular interest is the comparison with [Sar^1, Car^8]AT II where the presence of the highly hydrophobic carboranylalanine (Car) induced a much more decrease of relative affinity.

Our results are in good agreement with extensive structure-activity studies of AT II analogs which have shown that the residues in positions $1-7$ define the specificity, intensity and duration of the biological effect while the nature of the residue in position 8 establishes the hormone analog as agonist or antagonist.

Surprinsingly, substitution of CO by $P(C_6H_5)_3$, although dramatically increasing both hydrophobicity and bulkiness has little effect on relative affinity. The fact that no direct correlation is found between the size of the side chain in position 8 and antagonist activity is not in favor of any direct specific interaction between this side chain and the receptor. It could be postulated that its presence prevents the molecule from reaching its biologically agonist active conformation when the peptide is interacting with the receptor. This result is in good agreement with the study by Hsieh and Jorgensen[15] where it has been shown that a sterically locked phenylalanine in position 8 acts as a pure antagonist with good affinity. Preliminary experiments were also performed to use [Sar^1, Cym^8]AT II as a photoaffinity label assay for angiotensin receptor. Rabbit aorta strips were incubated with [Sar^1, Cym^8]AT II and irradiated during different periods of time. No desensibilization of the tissue to AT II could be detected although IR spectroscopy indicated the loss of carbonyl groups by [Sar^1, Cym^8]AT II. Monitoring of the photolysis by HPLC showed the formation of a major product, stable for several hours, which could explain the lack of reactivity with the receptor.

In conclusion, photosubstitution of cymantrenylalanine allows, by replacement of a carbonyl group by phosphine ligands to have access to a wide variety of analogs which can be used in classical solid phase synthesis. As shown with AT II, this offers the possibility of a new approach to structure-activity studies.

EXPERIMENTAL

Melting points were determined on a Buchi apparatus. Infrared spectra were recorded on a Nicolet FT-IR instrument. NMR spectra were recorded on a JEOL instrument operating at 60 MHz, TMS was used as internal standard. N^{α}-Boc protected amino-acids were obtained from Bachem, N^{α}-Boc-cymantrenylalanine was prepared as described previously[12]. Peptide samples for amino-acid analyses were hydrolyzed at 110°C during 24 h in 6M-HCl plus 0·2% phenol

in vacuum-sealed tubes. The amino-acid analyses were carried out on a Beckman 7 300 amino-acid analyzer.

N$^{\alpha}$-tert-Butyloxycarbonyl-cymantrenylalanine Methyl Ester (*III*)

N$^{\alpha}$-tert-Butyloxycarbonyl-cymantrenylalanine (8·7 g, 22·2 mmol) was dissolved in aqueous ethanol. The pH was adjusted to 7·0 with a 10% aqueous solution of Cs_2CO_3, then evaporated to dryness. The residue was stirred with methyl iodide (3·8 g, 26·7 mmol) in DMF (15 ml) for 12 h. Upon removal of the solvent, the residue was dissolved in ethyl acetate, washed ($NaHCO_3$, 5% aqueous), dried (Na_2SO_4), evaporated to an oily residue and crystallized from light petroleum: 7·2 g (80%), m.p. 86°C. ^{1}H NMR ($CDCl_3$): 5·2 m, 1 H (NH); 4·4–4·8 m, 4 H (Cym H); 4·2 m, 1 H (C$^{\alpha}$H); 3·75 s, 3 H (OCH_3); 2·75 m, 2 H (CH_2); 1·4 s, 9 H ($C(CH_3)_3$). For $C_{17}H_{20}MnNO_7$ (405·3) calculated: 50·38% C, 4·97% H, 13·55% Mn, 3·45% N; found: 50·32% C, 4·93% H, 13·61% Mn, 3·41% N.

2-(1-tert-Butyloxycarbonylamino-1-methoxycarbonyl)ethylcyclopentadienyl(dicarbonyl) (triphenylphosphine) Manganese *IV*

Methyl ester *III* (0·5 g, 1·2 mmol) was dissolved in 80 ml of benzene–cyclohexane (2 : 9) After addition of triphenylphosphine (0·435 g, 1·8 mmol), the solution was irradiated under a stream of nitrogen with a Hanovia high pressure mercury lamp, 150 W. The transformation was monitored by TLC (SiO_2, light petroleum–ether 7 : 3). After 10 min, the irradiation was stopped and the solution was evaporated to dryness. The residue was chromatographed on silica gel preparative layers (TLC conditions) to yield *IV*: 0·3 g (60%), m.p. 146°C. ^{1}H NMR ($CDCl_3$): 7·3 m, 15 H (A_2H); 5·2 m, 1 H (NH); 4·1–4·4 m, 4 H (Cym H); 4·2 m, 1 H (CH); 3·75 s, 3 H (OCH_3); 2·75 m, 2 H (CH_2); 1·4 s, 9 H ($C(CH_3)_3$). For $C_{34}H_{35}MnNO_6P$ (639·5) calculated: 63·8% C; 5·51% H; 8·69% Mn; 2·19% N; found: 62·94% C; 5·62% H; 8·66% Mn; 2·18% N.

Peptide Synthesis and Purification

N$^{\alpha}$-tert-Butyloxycarbonyl-cymantrenylalanine methyl ester *III* was treated with methanolic cesium hydroxide for 45 min. After evaporation, the residue was dissolved in DMF and reacted at 50°C with chloromethyl resin (Bio-Beads S-X1, 200–400 mesh, 1·25 mmol Cl/g). Yields of 0·5 to 0·8 mmol of Boc-Cym per gram of resin were obtained using of 1·5 fold excess of cesium salt. Amino-acid *IV* was treated in a similar manner. Coupling of the subsequent N$^{\alpha}$-Boc-protected amino-acids were performed following a protocol described elsewhere[12]. Side-chains were protected as follows: Arg(Tos), His(Tos), Tyr(2,6-dichlorobenzyl).

After completion of the last cycle, the peptides were cleaved from the resin and completely deprotected by treatment for one hour with HF in the presence of Me_2S (1 ml) and *p*-cresol (1 g) per gram of resin. After evaporation of HF, resins were extracted several times with 50% AcOH. After lyophilization, IR spectroscopy established the presence of the intact organometallic moieties.

Crude peptides were chromatographed in a first purification on Sephadex LH20 with DMF–0·1M-AcOH (1 : 3). The resulting products were further purified and diastereoisomers separated by preparative reversed-phase HPLC on a Magnum ODS-2 column (Whatman) using a linear gradient (0·1% TFA to 0·1% TFA–CH_3CN, 30 : 70). Purity was established by TLC and analytical reversed phase HPLC.

All peptides had the expected amino-acid composition except for the organometallic residues which were destroyed under these conditions. Their presence was assessed by IR spectroscopy.

Rabbit Aorta Strips

New Zealand rabbits weighting 1·5—2·0 kg were killed by stunning and exsanguination. The thoracic aorta were excised and immersed in oxygen saturated Krebs' solution. The aorta were helically cut into a 5 mm-large band which were suspended in 5 ml baths. A tension of 2 g was applied. Isomeric contractions of the aortae in response to angiotensin II, its agonists and antagonists were recorded with force displacement transducers. Relative affinities were compared to [Sar^1, Ala^8]AT II.

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