Synthesis of Biologically Active Retroenantiomers of Angiotensin Peptides

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The effect of the reversal of the direction of amide bonds in the peptide chain of angiotensin was determined by the synthesis and study of retroenantiomers of the following peptides: 1, [Val^5]angiotensin II (angiotensin); 2, [Suc^1]angiotensin (desamino-angiotensin); 3, [Ala^7]desamino-angiotensin; 4, [β-Ala^7]desamino-angiotensin. In all of these retroenantiomers, the N-terminal Phe residue was replaced by a benzylmalonyl moiety in order to maintain the topological features of angiotensin's C terminus which are important for biological activity. The separation of the diastereomeric peptides containing D- or L-benzylmalonyl residues was possible in the cases of the retroenantiomers of 1 and 2 but not in those of 3 and 4. The retroenantiomers of 1 and 2 were devoid of smooth muscle contracting activities, while those of 3 and 4 contracted the isolated guinea-pig ileum and rat uterus with activities ranging from 8 to 24%, when compared with the respective parent compounds. The results indicate that (a) the sense of the peptide bonds in angiotensin's backbone is not essential for activity, and (b) the Pro^7 residue in angiotensin is important for maintaining an "active" conformation of the molecule. The compounds reported in this paper are the first retroenantiomers of linear peptide hormones that have been shown to retain the biological activities of the parent compounds.

The study of the relationship between covalent structure and biological activities of angiotensin peptides has led to the conclusion that most of the side chains of angiotensin II are important for its smooth muscle contracting potency.[1] It is also known that, while a free terminal carboxyl group is essential for activity, the terminal amino group may be absent or blocked with retention of significant myotropic activity. Thus, the activities of [Suc^1]angiotensin II on the rat uterus, guinea-pig ileum, and rabbit aorta are respectively 47, 11, and 12% of the activity of angiotensin II.[2]

One aspect that has been neglected in the study of angiotensin's structure–activity relationships is the role of the peptide backbone. In order to have information on this matter, we have studied the effect of reversal of the direction of amide bonds in the peptide chain upon the biological activity of angiotensin peptides. For this purpose we have prepared retroenantiomers of [Val^5]angiotensin II (angiotensin), i.e., peptides containing D-amino acid residues in the reverse sequence as that of angiotensin. Such analogues are characterized by a reversed backbone with only minor changes in side-chain topology.[3,4] In the cases of the cyclic peptides or depsipeptides, the retroenantiomers of a gramicidin,[3] of enniatin B,[5] and of an antamanide[6] have been shown to be active, while the retroenantiomers of the linear peptides bradykinin,[7] α-melanotropin-(5–9)-pentapeptide,[8] tuftsin,[9] and desamino-gastrin-tetrapeptide amide[9] were devoid of biological activity.

Table I shows the amino acid sequence of angiotensin and of three analogues and also those of the respective retroenantiomers whose sequences are shown in the reverse of the conventional manner in order to facilitate comparison with the normal peptides. The latter shall be referred to as the "parent compounds" of the respective retroenantiomers.

We have initially prepared [1-benzylmalonyl]-*retro-enantio*-angiotensin (*retro-enantio*-angiotensin, 1) in which the benzylmalonyl residue served to introduce a carboxyl group at the "N terminus" to maintain, in the retroenantiomer, this important feature of angiotensin's C terminus. This analogue, however, contained another carboxyl group where angiotensin has its terminal amino group. To avoid the "amino group problem",[4] and since the amino group is not essential for activity, we have also synthesized [1-benzylmalonyl]-*retro-enantio*-desamino-angiotensin (*retro-enantio*-desamino-angiotensin, 2). This analogue still has an imperfection in its side-chain topology because of the presence of a prolyl residue, whose pyrrolidine ring imposes a restriction of the peptide backbone by the configuration of the "side chain".[3,4] Since analogues in which angiotensin's Pro^7 is replaced by other amino acids still retain some activity,[1] we have tried to avoid the "proline problem" by preparing [1-benzylmalonyl]-*retro-enantio*-[7-alanine]desamino-angiotensin ([Ala^2]-*retro-enantio*-desamino-angiotensin, 3) and [1-benzylmalonyl]-*retro-enantio*-[7-β-alanine]desamino-angiotensin ([β-Ala^2]-*retro-enantio*-desamino-angiotensin, 4). For comparison with these compounds, [Ala^7]desamino-angiotensin (5) and [β-Ala^7]desamino-angiotensin (6) were also synthesized.

Experimental Section

Melting points were determined with a Köfler apparatus (Zeiss-Wetzlar) and are uncorrected. Optical rotations were determined at 20 ± 5 °C with a Bellingham and Stanley "Pepol 60" photopolarimeter with a precision of 0.002 °C, and ir spectra were obtained with a Perkin-Elmer 337 spectrometer. ^{1}H NMR spectra were taken on a Varian T-60 instrument with Me_4Si as internal reference. TLC on 0.1-mm silica gel plates (Eastman "Chromagram", with fluorescent indicator) was run with the following solvent systems: A, *n*-BuOH–HOAc–H_2O (4:1:1); B, *n*-BuOH–EtOAc–HOAc–H_2O (1:1:1:1); C, *n*-BuOH–pyridine–HOAc–H_2O (30:20:6:24); D, EtOH–NH_4OH–H_2O (20:1:4); E, $CHCl_3$–MeOH–HOAc (85:10:5); F, $CHCl_3$–HOAc (95:5); G, acetone–HOAc (98:2). Peptides were located with ninhydrin, Pauly, and chlorine sprays and other compounds with bromocresol green or by uv fluorescence.

***tert*-Butyloxycarbonyl-D-amino Acids.** D-Amino acids were bought from Fluka AG and Calbiochem. The functional side chains were protected as follows: nitroarginine,[10] aspartic acid β-benzyl ester,[11] and *O*-benzyltyrosine.[12] Boc-Tos^{Im}-histidine was prepared as described by Fujii and Sakakibara.[13] The *tert*-butyloxycarbonyl (Boc) derivatives of the other amino acids were prepared by Schnabel's[14] method. All *tert*-butyloxycarbonyl-D-amino acids had melting points in agreement with the best literature values for the L enantiomers, and their infrared spectra were identical with those of the corresponding *tert*-butyloxycarbonyl-L-amino acids purchased from Bachem, Inc. They were homogeneous on TLC with solvents E, F, and G.

Benzylmalonic Acid *tert*-Butyl Monoester. To a mixture of 18.5 g of *t*-BuOH (0.25 mol) and 20 ml of *N,N*-dimethylaniline (0.16 mol), 11.5 g (0.05 mol) of benzylmalonyl chloride dissolved in $CHCl_3$ was slowly added, so that the temperature remained below 30 °C. The mixture was refluxed for 4 h, then cooled in an ice bath, and stirred while 75 ml of cold 6 N H_2SO_4 was added. The solution was then extracted with Et_2O and the extract washed with cold H_2SO_4, H_2O, 10% $NaHCO_3$, and saturated NaCl and then dried with anhydrous Na_2SO_4. The Et_2O was evaporated and the benzyl alcohol removed by vacuum distillation. The residue (15 g) migrated as a single spot on TLC with solvent system D (R_f 0.72): ir (film) 1725 (C=O), 1380, 1360 [$(CH_3)_3C$–],

Table I. Some Angiotensin Peptides and Their Retroenantiomers[a]

L-Asp → L-Arg → L-Val → L-Tyr → L-Val → L-His → L-Pro → L-Phe (angiotensin)
D-Asp ← D-Arg ← D-Val ← D-Tyr ← D-Val ← D-His ← D-Pro ← DL-BzlMal [*retro-enantio*-angiotensin (1)]
Suc → L-Arg → L-Val → L-Tyr → L-Val → L-His → L-Pro → L-Phe (desamino-angiotensin)
β-Ala ← D-Arg ← D-Val ← D-Tyr ← D-Val ← D-His ← D-Pro ← DL-BzlMal [*retro-enantio*-desamino-angiotensin (2)]
Suc → L-Arg → L-Val → L-Tyr → L-Val → L-His → L-Ala → L-Phe [[Ala^7]desamino-angiotensin (5)]
β-Ala ← D-Arg ← D-Val ← D-Tyr ← D-Val ← D-His ← D-Ala ← DL-BzlMal [[Ala^2]-*retro-enantio*-desamino-angiotensin (3)]
Suc → L-Arg → L-Val → L-Tyr → L-Val → L-His → β-Ala → L-Phe [[β-Ala^7]desamino-angiotensin (6)]
β-Ala ← D-Arg ← D-Val ← D-Tyr ← D-Val ← D-His ← β-Ala ← DL-BzlMal [[β-Ala^2]-*retro-enantio*-desamino-angiotensin (4)]

[a] The arrows indicate the sense of the -CO-NH- bond in the peptide backbone.

Table II. Physical Properties of Peptide Products

Compd	Yield,[a] %	Mp, °C	$[\alpha]^{25}D$,[b] deg	K[c]	R_f on TLC[a] A	B	C	Electrophoretic migration[a] pH 2.8 (R_{His})	pH 4.9 (R_{Arg})	pH 9.9 (R_{Picr})
1A[d]	6	200 dec[e]	+52.2[f]	0.69	0.44	0.55	0.85	0.30	-0.18	0.26
1B[d]	7	200 dec[e]	+42.3[f]	1.11	0.44	0.56	0.85	0.32	-0.18	0.27
2A[d]	12	249-251 dec	+81.1	0.65	0.39	0.55	0.85	0.46	0.00	0.43
2B[d]	10	245-257 dec	+66.6	1.06	0.40	0.52	0.83	0.46	0.00	0.42
3	10	243-245	+37.2	0.87	0.43	0.57	0.47	0.44	0.01	0.23
4	15	205 dec	+37.1	0.56	0.53	0.33	0.56	0.52	0.00	0.29
5	13	244-246	-42.3	0.59	0.40	0.63	0.80	0.43	0.05	0.14
6	16	243-245	-38.2	0.87	0.43	0.57	0.47	0.44	0.02	0.21

[a] See Experimental Section. [b] *c* 0.1, 1 N HOAc. [c] Countercurrent distribution with *n*-BuOH-HOAc-H_2O (4:1:5). [d] Diastereomers separated by countercurrent distribution are designated A and B. [e] Decomposition began at 200 °C, no melting up to 300 °C. [f] *c* 0.2, H_2O.

1250 and 1130 (-COC-), 835 [$(CH_3)_3CO$-], 750, 708 cm^{-1} (aromatic). This material (*tert*-butyl benzylmalonate, 0.05 mol), dissolved in 18 ml of EtOH, was semisaponified by adding 30.2 ml of carbonate-free 1.5 N KOH (45 mequiv), with stirring, until neutral reaction to phenolphthalein. The solution was filtered and then evaporated under vacuum. The residue was resuspended in H_2O, extracted with Et_2O, adjusted to pH 3.0 with 1 N H_2SO_4, and again extracted with Et_2O at 0 °C. The extract was dried with anhydrous Na_2SO_4, filtered, and evaporated, and the resulting oil crystallized as the potassium salt from hot benzene: yield 7.8 g; start decomposing between 120 and 125 °C; saponification index, 99% of theoretical; ir (free acid, film) same bands as the diester plus a broad band in the region 3000–2000 cm^{-1}, characteristic of -COOH; ^1H NMR (Me_2SO-d_6) δ 7.25 (s, 5, phenyl), 3.08 (d, 2, CH_2), 2.55 (t, 1, -CH), 1.25 (s, 9, $-CH_3$). Anal. ($C_{14}H_{17}O_4$) C, H. Attempts to separate the enantiomers by resolution of the brucine and quinine salts were unsuccessful, and the racemic mixture was used for the synthesis of peptides.

Peptide Syntheses. Peptides were prepared by the solid-phase method.[15] The C-terminal residue was esterified to a chloromethylated copolymer of styrene with 2% divinylbenzene (Schwarz BioResearch or Bio-Rad) as described by Merrifield,[16] and the degree of substitution was determined by amino acid analysis of a hydrolysate obtained by treating the amino acid–resin with HCl–PrOH (1:1) at 120 °C for 3 h.[17] Deprotection of amino groups was done with 30% TFA in $CHCl_3$ and neutralization with 10% TEA in CH_2Cl_2. Coupling was performed with 2.5 molar excess of the *tert*-butyloxycarbonylamino acid and DCCI in CH_2Cl_2 and monitored with the ninhydrin test.[18] In most cases coupling was completed in 2 h, but when the ninhydrin test remained positive after 6 h a recoupling cycle was performed, including EtOH washing, neutralization, and a repeated coupling, now with an added 2.5 molar excess of *N*-hydroxysuccinimide.

After the last coupling and deprotection the peptide was cleaved from the resin by treatment, at 0 °C for 60 min, with anhydrous HF containing 5% (v/v) anisole. After removal of HF and anisole by vacuum distillation, and washing with EtOAc, the peptides were extracted with glacial HOAc and freeze-dried.

The crude peptides were submitted to 200–800 transfers of countercurrent distribution in *n*-BuOH–HOAc–H_2O (4:1:5), using a quantitative Pauly reaction[15c] for detection. The distribution coefficient (K) for each peptide is shown in Table II. Further purification was accomplished by ion-exchange chromatography on carboxymethylcellulose with a linear elution gradient between 0.1 M NH_4OAc (pH 4.7) and 3.5 M HOAc. When necessary, the peptide was further purified by chromatography on a Dowex 1-X2 (200–400 mesh) column with an elution gradient from 0.01 M NH_4OAc (pH 8) to 0.1 M NH_4OAc (pH 6) or on a Dowex 50-X2 (200–400 mesh) with an NH_4OAc gradient from 0.01 (pH 5.5) to 0.1 M (pH 8.0).

The chromatography eluates containing the peptides were lyophilized, until constant weight, to remove NH_4OAc. Each peptide was purified until the following criteria for purity were met. (a) The amino acid analysis of the 72-h acid hydrolysates (6 N HCl, 120 °C), performed on a Beckman Model 120 C analyzer, yielded a molar ratio within 4% of the theoretical value for all amino acid residues. (b) The peptide content, obtained from amino acid analysis, agreed within 1% with that obtained from the absorbance of aqueous solutions at 275 nm, taking the value of 1375 for the molar extinction coefficient. (c) Only one spot was detected on TLC of a 0.1-μmol sample with solvents A, B, and C. (d) Only one component, with the expected mobility, could be detected after paper electrophoresis of a 0.1-μmol sample at 1000 V for 60 min in three different buffer systems: 1 M HOAc (pH 2.8), 0.1 M pyridine acetate (pH 4.9), and 0.2 M sodium carbonate–bicarbonate (pH 9.9). The electrophoretic mobility of the peptide is expressed in Table II by the ratio of its migration to that of a simultaneously run standard. At pH 2.8 the migrations are relative to histidine (R_{His}), at pH 4.9 to arginine (R_{Arg}), and at pH 9.9 to picric acid (R_{Picr}) and they were given a negative sign when peptide and standard had opposite charges. The yields of the syntheses were calculated from the peptide content of the purified product (determined by amino acid analysis), related to the amount of *tert*-butyloxycarbonylamino acid initially esterified to the resin. The low yields, shown in Table II, reflect the policy of stressing purity rather than yield when pooling the fractions of countercurrent distribution and chromatography.

In the peptides containing benzylmalonyl residues, the presence of benzylmalonic acid in the hydrolysates was qualitatively determined by identification of the hydrocinnamic acid resulting from decarboxylation at high temperature. The hydrolysates obtained by treatment with 6 N HCl at 120 °C for 72 h were evaporated to dryness, dissolved in H_2O, and extracted with Et_2O. The aqueous phase and the extract were submitted to TLC with solvent D and revealed with ninhydrin, uv fluorescence, and bromocresol green. The amino acids remained in the aqueous phase and hydrocinnamic acid (R_f 0.69) was found in the Et_2O extract.

Since the racemic benzylmalonic acid derivative was employed in the syntheses of the retroenantiomers (compounds 1–4), the crude products were assumed to contain a mixture of equal amounts of the two diastereomeric peptides. In the cases of *retro-enantio*-angiotensin and *retro-enantio*-desamino-angiotensin, it was possible to separate, by countercurrent distribution, two

Table III. Smooth Muscle Contracting Activity of Angiotensin Analogues Modified at Residue 7[a]

Peptide	Guinea-pig ileum	Rat uterus
[Ala^7]desamino-angiotensin	0.7 ± 0.1	0.4 ± 0.1
[β-Ala^7]desamino-angiotensin	0.08 ± 0.01	0.02 ± 0.1
[MeAla^7]angiotensin[b]	4.0 ± 0.1	
[3-Hyp^7]angiotensin[c]		10.0

[a] Activities ± one standard deviation are expressed in percentage of the activity of the corresponding peptide with Pro^7. [b] Reference 22. [c] Reference 1.

fractions (A and B) that had the same amino acid composition (including the presence of benzylmalonic acid) and had identical physical properties (Table II) except for their optical rotations and partition coefficients in *n*-BuOH–HOAc–H_2O. These two fractions were assumed to be the isolated diastereomers and assayed separately. Attempts to resolve diastereomers during the purification of compounds 3 and 4 were not successful. Since these purified products had homogeneous behavior on countercurrent distribution and chromatography, it was assumed that they contain equal amounts of the two isomers in each case.

The angiotensin and desamino-angiotensin preparations used in this study have been previously described.[2,19] The physical properties of the other compounds that were prepared are shown in Table II.

Bioassays. The isolated guinea-pig ileum[20] and rat uterus[21] preparations were done as described earlier. The bath volume was 5 ml, the media were aerated with a stream of air, and the isotonic contractions were recorded under a 1-g load with sixfold magnification. The evaluations of biological activity were made by direct comparison with a standard peptide through four-point assays. These comparisons were made on a molar basis, taking into account the peptide content of each preparation, as determined by amino acid analysis and absorbance at 275 nm.

Results and Discussion

Table III shows the effect of replacements at residue 7 on the smooth muscle contracting activities of angiotensin analogues. The importance of the Pro^7 residue for angiotensin's biological activities has been attributed[1] to one of the following properties being important for maintaining a proper spacial relationship between the C-terminal residues or for "productive" binding to the cell receptor: (a) the rigidity of the peptide chain at the pyrrolidine ring; (b) the lack of a potential H-bonding site in the prolyl residue; (c) a preference for cis His-Pro peptide bonds. This last possibility is not supported by ^{13}C NMR data indicating 100% trans conformation at that bond.[23] Another possibility is that the prolyl side chain might participate in a hydrophobic interaction that could be important for activity, as is suggested by the tenfold reduction of oxytocic activity observed in [3-Hyp^7]angiotensin. However, a roughly similar loss of activity on the guinea-pig ileum was observed in [MeAla^7]angiotensin, whose *N*-methylalanine residue would have hydrophobic properties similar to those of proline. This analogue is characterized by the loss of the rigidity imposed on residue 7 by the pyrrolidine ring, although *N*-methylalanine still restricts its freedom by a factor of 2 (relative to alanine), also confining the previous residue to positive values of ψ.[24] A greater increase in the freedom of rotation is obtained in [Ala^7]angiotensin, whose activity is further reduced by one order or magnitude, relative to [MeAla^7]angiotensin. This reduction might be caused either by the increase in rotational freedom or by the introduction of H-bonding potentiality at residue 7. However, further evidence in favor of Pro^7's role in positioning the C-terminal residues of angiotensin in an "active" configuration comes from the fact that [β-Ala^7]angiotensin's activities are one order of magnitude smaller than those of [Ala^7]angiotensin. Indeed, a much greater conformational freedom would be expected in the C-terminal portion of the β-Ala^7 analogue.

Table IV. Smooth Muscle Contracting Activity of Retroenantiomers of Desamino-angiotensin and 7-Substituted Analogues

	Peptide	Activity[a] Guinea-pig ileum	Activity[a] Rat uterus
1	*retro-enantio*-Angiotensin (A and B)	<0.01	<0.05
2	*retro-enantio*-Desamino-angiotensin (A and B)	<0.01	<0.05
3	[Ala^2]-*retro-enantio*-desamino-angiotensin	8.5 ± 0.3	7.7 ± 0.5
4	[β-Ala^2]-*retro-enantio*-desamino-angiotensin	24.2 ± 1.0	8.3 ± 0.4

[a] Activities ± SD are in percentage of the activities of the respective parent compounds.

The effect of reversal of the peptide backbone on the smooth muscle contracting activities is indicated by the results of the assays of compounds 1–4 (Table IV). No agonistic or antagonistic activity could be detected in either of the two fractions (diastereomers) of *retro-enantio*-angiotensin or *retro-enantio*-desamino-angiotensin, but compounds 3 and 4 had quite significant agonistic activities when compared with those of the corresponding parent compounds. These activities are even greater if it is taken into account that what was assayed was probably a 1:1 mixture of the two diastereomers in each case. Since the change of configuration of the phenylalanine residue in angiotensin results in 100-fold loss of activity,[25] it is probable that only the diastereomers containing the D enantiomer of the benzylmalonyl residue are responsible for the smooth muscle contracting activities. Therefore, the biological potencies of these diastereomers should be twice as great as the values shown on Table IV. These relatively large activities of the retroenantiomers indicate that the peptide groups of the backbone do not participate in interactions that are essential for the manifestation of myotropic activity. Compounds 3 and 4 are, to our knowledge, the first reported retroenantiomers of linear peptides that retain some of the biological activity of the parent compounds,[9] although their activity is very small when compared to desamino-angiotensin (see Table III). It should be emphasized, however, that these compounds behaved as typical angiotensin-like peptides, being active in concentrations of the order of 10^{-5}–10^{-4} M.

Although the lack of activity of *retro-enantio*-angiotensin may be explained by the presence of a carboxyl where angiotensin has an amino group, the inactivity of *retro-enantio*-desamino-angiotensin reinforces the argument for the importance of the conformational restrictions due to the Pro^7 residue in angiotensin. It seems that the difference in side-chain topology due to the prolyl residue in *retro-enantio*-desamino-angiotensin, compared to desamino-angiotensin, is enough to abolish activity. On the other hand, in the analogues in which Pro^7 is replaced by Ala or β-Ala, the side-chain topology is essentially maintained in the retroenantiomers, which also retained a considerable fraction of the small activities of the respective parent compounds.

The different countercurrent distribution behavior of the diastereomers of the two retroenantiomers containing proline (1 and 2), not observed with the other compounds (3 and 4), is a strong indication that there are intramolecular interactions involving the C-terminal end of angiotensin which are in agreement with other physi-

cochemical properties of angiotensin peptides[19b,23,26] and, besides determining the solubility properties responsible for the countercurrent distribution behavior, may also be important for the biological activities. This would explain the great loss of activity on going from angiotensin to [MeAla^7]angiotensin to [Ala^7]angiotensin to [β-Ala^7]-angiotensin. It would also explain why the Pro^2-containing retroenantiomers are inactive, while those containing more flexible residues retain a substantial portion of the activities of the respective parent compounds.

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Protein-Binding Polyhedral Boranes. 3[1]

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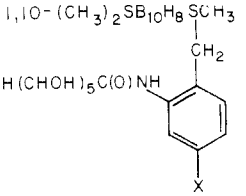
A series of polyhedral borane derivatives containing protein-binding functional groups has been synthesized. Problems encountered in earlier studies (low incorporation levels, gross precipitation of conjugates) have been overcome by including a water-solubilizing gluconamide group in the structure. This modification has allowed high levels of boron to be covalently bound to HGG, forming a completely water-soluble conjugate.

In anticipation of the expected isolation of tumor-specific antibodies,[2] several research groups have investigated the attachment of boron-containing moieties to proteins.[1,3-5] These investigations aim to demonstrate the feasibility of transporting boron into neoplasms, via antibody carriers, thereby providing the basis for therapeutic neutron-capture irradiation. Toward this end, we have reported the synthesis of a number of polyhedral borane derivatives containing protein-binding functional groups and their incorporation into bovine serum albumin (BSA) and human γ-globulin (HGG).[1] These results have indicated that proteins may be appropriately modified by our method. Unfortunately, the derivatives which were used in those initial experiments reduced the aqueous solubility of the proteins, especially HGG, so severely that only a small number of boron-containing labels could be incorporated prior to denaturation of the conjugate.

Chart I. Structures of the Protein Binding Polyhedral Borane Derivatives



1a, X = NH_2
b, X = NCS
c, X = CO_2H

In seeking to overcome this problem, we have undertaken the attachment of a water-solubilizing group onto the appropriate polyhedral borane derivatives. For reasons