# Synthesis of Ala-Pro-Gly-[Ile$^3$,Val$^5$]angiotensin II Isolated from the Skin of the Australian Frog *Crinia georgiana*

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Ala-Pro-Gly-[Ile$^3$,Val$^5$]angiotensin II was synthesized by Merrifield's solid-phase procedure. The peptide was purified by chromatography on successive columns of anion-exchange resin, Sephadex G-25 and SP-Sephadex C-25; its homogeneity was determined by degradation with $\alpha$-chymotrypsin, ionophoresis, thin-layer chromatography, and high-pressure liquid chromatography (HPLC). The dansyl derivative of this angiotensin has the same chromatographic behavior (TLC) as the dansyl undecapeptide, "*Crinia* angiotensin II", isolated from the skin of the Australian frog *Crinia georgiana*. The pressor activity of the synthetic undecapeptide (in rats anesthetized with sodium amytal, followed by treatment with a solution of hexamethonium chloride containing polyvinylpyrrolidone, and vagatomy) was 90.6 ± 4.99% ($n$ = 26, 7 rats) of that of [Ile$^5$]angiotensin II (human angiotensin II).

Erspamer et al.[1] reported angiotensin-like radioimmunoreactivity in the skin of some species belonging to the genus *Crinia*. Of all the numerous species studied, the Australian frog, *Crinia georgiana*, contained the highest quantity of the pressor substance in its skin extract (130–155 $\mu$g/g of dried skin). On the basis of its amino acid composition and its mode of degradation with TPCK-trypsin and $\gamma$-chymotrypsin, the angiotensin-like peptide from the skin of *Crinia georgiana* has been identified as an undecapeptide and designated as "*Crinia* angiotensin II".[2]

A comparison of the structure of *Crinia* angiotensin II with angiotensin II of mammalian and nonmammalian origin indicates that the amino terminus in *Crinia* angiotensin II has been elongated with a tripeptide (Ala-Pro-Gly) and that valine (position 3) and isoleucine (position 5) residues have been interchanged (Figure 1). This unique structure of *Crinia* angiotensin is of interest in phylogenetic studies and also in investigations of the functions of angiotensins in the skin and its involvement in sodium metabolism or in the regulation of blood pressure. The present paper reports the synthesis of *Crinia* angiotensin II to provide evidence for its structure and to study its pressor activity.

Ala-Pro-Gly-[Ile$^3$,Val$^5$]angiotensin II was synthesized by the solid-phase procedure,[3] and the pressor activity was determined on vagotomized, ganglion-blocked rats (see Experimental Section).

## Results and Discussion

The native *Crinia* angiotensin II, after dansylation, was cochromatographed with the dansylated derivative of Ala-Pro-Gly-[Ile$^3$,Val$^5$]angiotensin II. Either angiotensin showed an identical behavior on the thin-layer chromatogram. Rat pressor assays indicated that *Crinia* angiotensin II has 90.6 ± 4.99% ($n$ = 26) of the pressor activity of human angiotensin II, [Asp$^1$,Ile$^5$]angiotensin II.

So far, all the angiotensins II that have ever been isolated from plasma and characterized from mammals, avians, reptiles, and teleosts are octapeptides (Figure 1). Angiotensin II isolated from the plasma of human, horse, pig, mice, rat, rabbit, and dog contains valine in position 3 and isoleucine in position 5. The hormone isolated from all other species is substituted with valine in both these positions, 3 and 5.[4,5] *Crinia* angiotensin II is the first example of species variation in which substituents in position 3 and 5 have been interchanged.

It has been observed that the main peak of the peptide from the plasma of frog (*Rana catesbeiana*) is chromatographically similar to that of snake angiotensin. The $NH_2$ terminus of the snake plasma peptide is also acylated with an as yet unidentified moiety.[4,5] There is some evidence to suggest that the tissue angiotensins (e.g., produced by the corpuscles of Stannius) are structurally different from those of renal origin in some species such as carp[6] and the Japanese goosefish.[7]

It is possible that the functions of the renin–angiotensin system in the skin of reptiles and amphibians may be different than in their plasma. However, the known biological properties of *Crinia* angiotensin II appear to be similar to those of conventional angiotensin II, with the exception of the isolated guinea pig gallbladder on which it is 2–3 times more potent than [Asn$^1$,Val$^5$]angiotensin II.[8] Further investigations with *Crinia* angiotensin II may be useful in studying the changing role of renin–angiotensin in tissues and plasma of aquatic and land-living animals.

## Experimental Section

*tert*-Butyloxycarbonyl-protected amino acids were purchased from Bachem Inc., Torrence, CA. Ala-Pro-Gly-[Ile$^3$,Val$^5$]angiotensin II was synthesized by the solid-phase procedure.[3] The protocol used for the synthesis was similar to the one previously

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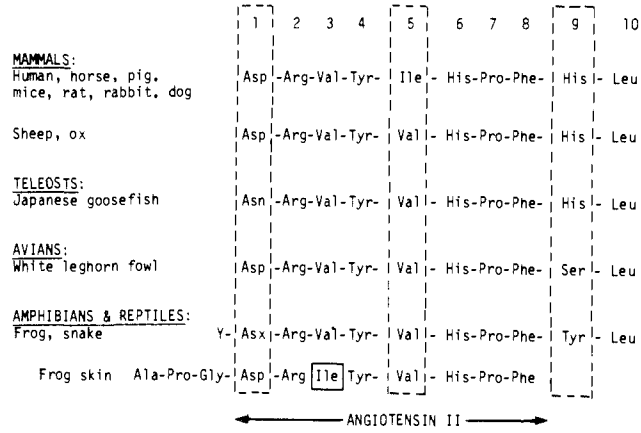


**Figure 1.** Variation in the structure of angiotensin during the course of evolution: Asx = Asp or Asn; Y = unidentified acyl moiety.

described by Khosla et al.[9] for the synthesis of analogues of angiotensin II. Side-chain functional protecting groups employed were Asp(β-Bzl), Arg($NO_2$), Tyr(2,6-$Cl_2$Bzl), and His(Cbz); α-amino groups were blocked with the *tert*-butyloxycarbonyl group. For quantitative coupling of Boc-valine, a preformed active ester with a 1 M excess of 1-hydroxybenzotriazole was used.[10] At the end of the synthesis, the peptide was cleaved from the polymer and partially deblocked with HBr/$CF_3CO_2H$ at room temperature.[11] Complete deblocking of the peptide was carried out by hydrogenolysis over 5% palladium/$BaSO_4$ in a mixture of MeOH/AcOH/$H_2O$ (5:1:1) under 2 atm of $H_2$ for 36–48 h in a Parr hydrogenation apparatus. Amino acid analysis of the crude product was carried out to ensure deblocking of side-chain protecting groups.

The crude product was purified on a column of Bio-Rad anion-exchange resin (AG-1×2, 200–400 mesh, acetate form) by eluting with 0.1 M ammonium acetate buffer (pH 8.5) containing 5% *n*-PrOH.[12] The column was eluted at the rate of 50 mL/h and the fractions giving a Pauly-positive reaction were chromatographed on cellulose TLC using 1-BuOH/pyridine/$H_2O$ (10:2:5) as the solvent. Fractions with the same $R_f$ values were pooled, evaporated to a small volume, and lyophilized. One milligram from each of four pools of fractions was hydrolyzed for amino acid analysis. Fractions which gave correct amino acid analyses were then rechromatographed in the same manner on successive columns of the following adsorbents and solvent systems, respectively: (a) Sephadex G-25, 1-BuOH/pyridine/$H_2O$ (10:2:5), upper phase; (b) Sephadex LH-20, 1-PrOH/AcOH/$H_2O$ (67:33:1.8), upper phase; (c) SP-Sephadex C-25 (40–120 μm) by a modification of the procedure reported by Erspamer et al.[2] Activated SP-Sephadex was equilibrated successively with 0.1% bovine albumin (Sigma Chemical Co., no. A-7511), 3 M $NH_4OAc$ (pH 5), and 0.05 M $NH_4OAc$ (pH 3). A solution of the sample (61 mg) in the latter buffer was applied to the column (1.5 × 75 cm), and the column was eluted by using a linear gradient of $NH_4OAc$ buffers (150 mL, 0.05 M $NH_4OAc$, pH 3, as the initial buffer and 150 mL, 0.5 M $NH_4OAc$, pH 6.5, as the gradient-forming buffer). This way, 50 fractions of 6 mL each were collected. Fractions 35 and 36 were combined and evaporated, and the residue was desalted by gel filtration on a column of Sephadex G-10 to yield 40 mg of the product. Fractions in the column chromatography were cut with emphasis on purity rather than on yield, and no attempt was made to rechromatograph the minor fractions for identification purposes.

The homogeneity of the peptide was determined by (a) a thin-layer chromatography in solvents of different pH, (b) ionophoresis at pH 2.3 and 8.6, (c) high-pressure liquid chromatography (HPLC), (d) amino acid analysis, (e) degradation with α-chymotrypsin, and (f) TLC of dansyl peptide.

TLC (cellulose, E. Merck) $R_f$ 0.50 [1-butanol/acetic acid/water (4:1:5)], 0.21 [1-butanol/pyridine/$H_2O$ (10:2:5)], 0.38 [1-butanol/pyridine/$H_2O$ (65:35:35)], 0.70 [1-butanol/acetic acid/water/pyridine (30:6:24:20)], 0.89 [1-propanol/$H_2O$ (2:1)].

Ionophoresis, carried on filter paper strips (Schleicher & Schuell, 2043A) in a Beckman electrophoresis cell (Model R, Series D) at 400 V using 1 M AcOH, gave a 0.75-fold relative electrophoretic mobility for Ala-Pro-Gly-[$Ile^3$,$Val^5$]angiotensin II, as compared to histidine. The corresponding ionophoretic mobility of this peptide in Beckman barbiturate buffer $B_2$ (pH 8.6) was 1.04 as compared to histidine. Detection of the compounds on TLC plates and on ionophoresis filter paper strips was carried out by spraying with Pauly's and Fluoram (30 mg in 100 mL of acetone) reagents.

Reversed-phase HPLC was carried out on Waters Associates liquid chromatograph (Model 6000A solvent delivery system and U6K injector) fitted with a data module and a variable-wavelength detector (Model 450) set at a fixed wavelength of 280 nm and at 0.02 AUFS. The peptide (in concentrations ranging from 10 to 25 μg) was dissolved in the eluting solvent system and chromatographed at room temperature on a μ-Bondapak $C_{18}$ column [$Si(CH_2)_{17}CH_3$, 3.9 mm i.d. × 30 cm] using MeOH/$H_2O$/$CF_3COOH$ (450:550:1, v/v) as the solvent system. The flow rate of the eluant was 1 mL/min, the chart speed was 0.5 cm/min, and the pressure was 1000 psi. The peptide showed a single peak at 8.58 min. Since the eluting solvent system is volatile, the compound was recovered by evaporation in a rotary evaporator followed by lyophilization.

The peptide was hydrolyzed in a sealed tube in 6 M HCl at 110 °C in the presence of 0.1 mL of 90% aqueous phenol. Amino acid analysis, performed on a Model MM-100 Glenco amino acid analyzer, gave the following ratios: Ala, 1.11; Pro, 1.99; Gly, 1.10; Asp, 0.99; Arg, 0.97; Val, 1.0; Tyr, 0.91; Ile, 0.93; His, 0.92; Phe, 0.92.

Specific optical rotation, as determined on a Perkin-Elmer polarimeter Model 141, equipped with a digital readout, was $[\alpha]^{20}_D$ −90.9° (*c* 0.2, 5 N acetic acid).

Enzymatic degradation was carried out with α-chymotrypsin (beef pancreas, three times crystallized, Nutritional Biochemical Corp.), 1 mg of the peptide was dissolved in 0.3 mL of ammonium acetate buffer (0.1 mol, pH 7.8), and an aliquot was spotted on TLC (cellulose plates). Chymotrypsin (0.2 mg suspended in 0.2 mL of ammonium acetate buffer, pH 8.5) was added to the above solution, and the mixture was incubated at 37 °C for 1 h. Acetic acid (0.2 mL) was added on the mixture was lyophilized. TLC in solvent systems of different pH indicated that after incubation with chymotrypsin the peptide was degraded, as expected, into two components. The presence of an additional spot would indicate an impurity.

The peptide (5 μg) was dansylated by dissolving it in 0.1 N $NEt_3$ (10 μL), followed by the addition of a solution of dansyl chloride in dioxane (0.5%; 10 μL). The tube was tightly plugged, stored overnight in the refrigerator in the dark, and then lyophilized. To the residue was added 5 μL of 90% formic acid and 50 μL of water. After 2 h at room temperature, the solution was again evaporated to dryness with a nitrogen stream. This way the labile dansyl moiety was removed from the imidazole ring. A comparison of this product with the dansyl derivative of native angiotensin was carried out on TLC (HPTLC, Merck): $R_f$ 0.53 [1-butanol/acetic acid/water (4:1:5)], 0.19 [2-propanol/methyl acetate/28% $NH_4OH$ (9:7:4)]. The dansyl derivatives of both the native and the synthetic undecapeptides showed the same chromatographic behavior.

**Pressor Activity.** Pressor activity was determined on anesthetized, ganglion-blocked, vagotomized rats. The protocol used was the same as reported by Pickens et al.,[13] except that, instead of pentolinium chloride, a 1-mL solution of hexamethonium chloride containing polyvinylpyrrolidone was injected as a ganglion-blocking agent. The latter solution was prepared by dis-

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solving 20 g of polyvinylpyrrolidone and 500 mg hexamethonium chloride in 100 mL of $H_2O$. *Crinia* angiotensin showed 90.6 ± 4.99% ($n$ = 26, 7 rats) of the pressor activity of [Ile$^5$]angiotensin II.

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# Synthesis of 5′-Thymidinyl Bis(1-aziridinyl)phosphinates as Antineoplastic Agents

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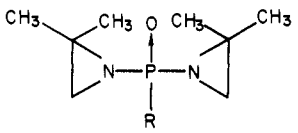
Reaction of 3′-acetylthymidine with phosphorus oxychloride in trimethyl phosphate yielded the phosphorodichloridate **5**, which was subsequently reacted with aziridine or 2,2-dimethylaziridine to give compounds **6** and **7**, respectively. The 2,2-dimethylaziridine derivative **7** was considerably more active than **6** against leukemias L1210 and P-388 in mice but less active than the previously synthesized, simpler phosphinate derivatives **2** and **3**. It appears that the thymidine moiety did not enable these compounds to use the nucleoside transport mechanism of the cells and also failed to increase the selectivity of the 2,2-dimethylaziridine analogues by interference with their binding to cholinesterase. Compound **7** strongly inhibited horse serum cholinesterase, while **6** was inactive.

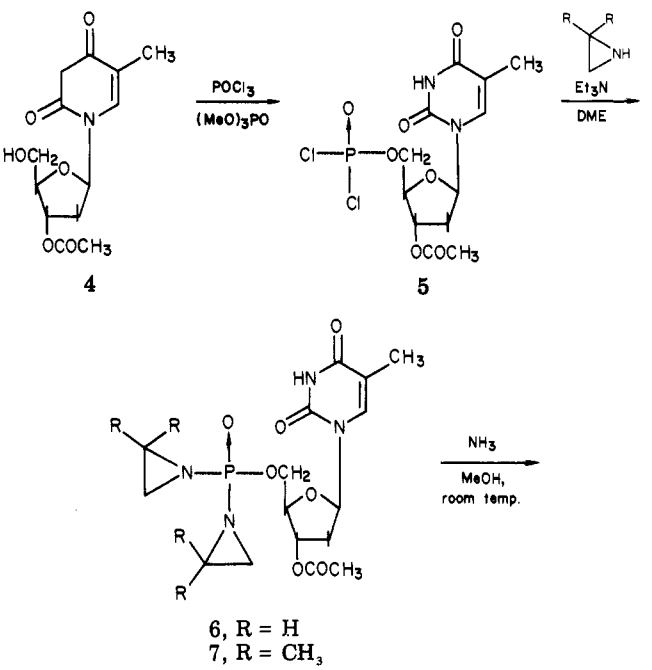
Several compounds termed "dual antagonists"[1] containing the bis(2,2-dimethylaziridinyl)phosphinyl moiety showed significant antitumor activities[2,3] and, in addition, demonstrated some marked radiation potentiating,[4-6] as well as cholinesterase inhibitory,[7] effects which were attributed to the potential phosphorylating activities of their transient hydrolysis products having five-membered oxaphospholidine ring moieties.[8] Characteristically, the 2,2-dimethyl-substituted phosphoraziridines (**1–3**) showed



**1** (AB-132),[1,2] R = $NHCO_2C_2H_5$
**2** (AB-163),[2] R = $OC_2H_5$
**3** (AB-182),[3] R = $ONHCO_2C_2H_5$

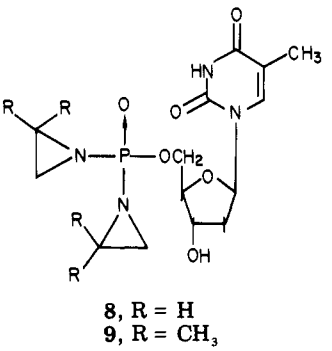
relatively little or no hematologic toxicity in animal experiments as well as in the clinical studies;[4-9] instead, gastrointestinal and CNS toxicities related to cholinesterase inhibition appeared to be their dose-limiting side effects.

**Scheme I**



**4** **5**

**6**, R = H
**7**, R = $CH_3$



**8**, R = H
**9**, R = $CH_3$

In an effort to increase the selectivity of action of these agents on the DNA template,[8] we decided to link their common reactive moiety to the 5′ position of thymidine. Compounds **7** and **9** (Scheme I) represent the desired "DNA-targeted" 2,2-dimethylphosphoraziridines, while compounds **6** and **8** are the corresponding ring-C-unsub-

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