tetralin system. Also, in the latter system, they favored exclusively p-p-phenol coupling producing a single dienone[5,6]. Further work should shed more light on the reagents most favorable for construction of the spiro system containing an indan moiety.

1 Acknowledgment. Supported in part by the Research Institute of Pharmaceutical Sciences, School of Pharmacy, University of Mississippi.

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Amino acid composition and sequence of crinia-angiotensin, an angiotensin II-like endecapeptide from the skin of the Australian frog *Crinia georgiana*[1]

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Summary. Methanol extracts of the skin of the Australian amphibian *Crinia georgiana* contain large amounts of crinia-angiotensin II, a new angiotensin II-like peptide. This differs sharply from the conventional octapeptide angiotensins II in having attached the tripeptide Ala-Pro-Gly- to the N-terminus, and having an Ile residue substituted for the Val residue at position 6 from the C-terminus. Small amounts of angiotensin-like peptides have been traced, by radioimmunoassay, in skin extracts of some other *Crinia* species.

A new peptide, which can be included in the angiotensin family, has been traced in the skin of the Australian frog *Crinia georgiana*. The peptide, called crinia-angiotensin, has been isolated in a pure form and its structure identified as follows:

Ala-Pro-Gly-Asp-Arg-Ile-Tyr-Val-His-Pro-Phe

It may be seen that crinia-angiotensin strikingly differs from all other known angiotensins (ox, horse, fowl, snake angiotensins) in that it has a tripeptide (Ala-Pro-Gly-) attached to the N-terminal Asp residue of the conventional angiotensins, and in that a Ile residue is substituted for the usual Val residue at position 6 from the C-terminus.

Materials. The dried skins of 357 specimens of *Crinia georgiana*, collected in Western Australia during the period 1973–1974 were used in this study. The material weighed 26.1 g (average 0.073 g per dry skin). The skins, removed from the frogs immediately after killing and dried in the shade, were subjected to 2 successive extractions with 20 parts (w/v) of 80% methanol, each extraction lasting 3–4 days. The extracts were mixed and filtered.

Isolation procedure. An aliquot of extract corresponding to 20 g of dried skins was evaporated to dryness and the residue taken up in water plus 99% ethanol to give a final concentration of 95%. After standing, the limpid supernatant was passed through a column of 170 g alkaline alumine, which was eluted with ethanol-water mixtures of decreasing concentrations of ethanol, each of 200 ml. The peak of angiotensin-like bioactivity (guinea-pig ileum preparation) appeared in the 60% ethanol eluates. Ethanol eluates 60_2 and 60_3 were mixed and used for this study.

One-tenth of the above active eluates (= 2 g dried skin) was evaporated, the residue taken up in 4 ml distilled water adjusted to pH 3 with formic acid, and then passed through a column (9×750 mm) of SP-Sephadex (NH_4^+ form). Linear gradient elution was carried out (fractions of 5 ml) with 150 ml H_2O and 150 ml 0.5 N $HCOONH_4$ (pH 6.5). 3.5×10^{-7} moles of pure peptide were recovered in fractions 24–26.

Structure. Amino acid composition, after the usual acid hydrolysis, was as follows: His 1, Arg 1, Asp 1, Pro 2, Gly 1, Ala 1, Val 1, Ile 1, Tyr 1 and Phe 1.

2 µg of peptide, dissolved in 50 µl of 0.1 N triethylamine bicarbonate (pH 8.5), were digested at 37 °C, overnight, by adding 5 µl of TPCK-trypsin solution (1 mg/ml). The reaction mixture was dansylated. 2 dansylated fragments could be separated by TLC on silica gel, developed first with acetone and then with n-butanol : acetic acid : water (4:1:5).

After acid hydrolysis (6 N HCl, 110 °C for 24 h), the 2 fragments showed the following amino acid composition: DNS-Ala (Arg, Asp, Pro, Gly) and DNS-Ile (His, Pro, Val, Phe, O-DNS-Tyr).

Repeating the above treatment with β-chymotrypsin produced similarly 2 dansylated fragments: DNS-Ala (Arg, Asp, Pro, Gly, Ile, O-DNS-Tyr) and DNS-Val (His, Pro, Phe).

This 2nd fragment was identical with that obtainable from Val[5]-angiotensin II: DNS-Val-His-Pro-Phe.

Thus, the following partial sequence of crinia-angiotensin may be suggested:

Ala-(Asp, Pro, Gly) Arg-Ile-Tyr-Val-His-Pro-Phe

5 µg of peptide were treated with sodium in liquid ammonia as described by Araki et al.[2]. After reduction, the mixture was hydrolysed with 6 N HCl and the hydrolysate was analysed, showing lack of the Ala and His residues. This strongly suggests the presence of Ala-Pro and His-Pro bonds in the sequence.

The dansyl Edman procedure was then performed on 10 nmoles of the intact peptide, obtaining the sequence

Ala-Pro-Gly-Asx-Arg-Ile-Tyr-Val-His-Pro-Phe

In the last step of degradation, dansyl Phe was detected without acid hydrolysis. This suggests that the C-terminal position of the peptide was not blocked.

Finally, 2 µg of peptide dissolved in 50 µl of 0.1 N triethylamine bicarbonate (pH 8.5) were digested at 25 °C, overnight, by adding 10 µl of amino peptidase M solution (1 mg/ml). The enzymatic hydrolysate was directly analysed, showing the presence of an equimolar amount of aspartic acid.

The angiotensin-like radioimmunoreactivity (expressed as Ile^5-angiotensin II) varied, in skin extracts from different batches of *Crinia georgiana,* between 130 and 550 µg per g dried skin; that of skin extracts of other *Crinia* species was as follows: *Cr. glauerti,* 5–10 µg/g; *Cr. leai,* 0.8–2 µg/g; *Cr. tinnula,* < 0.1 µg/g; *Cr. pseudoinsignifera,* < 0.1 µg/g; *Cr. subinsignifera,* < 0.1 µg/g; *Cr. signifera,* < 0.1 µg/g; *Metacrinia nicholsi,* 0.9–1.0 µg/g.

Angiotensin-like immunoreactivity was probably present also in skin extracts of *Litoria adelaidensis* (0.7–0.8 µg/g dried skin), while lacking (< 10 ng/g) in numerous other Australian frog species.

Kits for Ile^5-angiotensin II were obtained from Sorin, Saluggia, Italy.

Parallel bioassay of crinia-angiotensin II and of the conventional angiotensins II is in progress. On the usual isolated smooth muscle preparations, the activity of crinia-angiotensin was similar, also from a quantitative point of view, to that of Val^5-angiotensin-II-Asp^1-β-amide (Hypertensin® Ciba).

1 Supported by grants from the Consiglio Nazionale delle Ricerche, Italy, and from the Ministry of Education, Japan.

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Occurrence of Hyp^3-bradykinin in methanol extracts of the skin of the South African leptodactylid frog *Heleophryne purcelli*[1]

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Summary. Methanol extracts of the skin of the South African amphibians belonging to the genus *Heleophryne (H. purcelli purcelli, H. purcelli depressa, H. purcelli orientalis, H. natalensis)* contain large amounts (20–500 µg/g fresh tissue) of Hyp^3-bradykinin.

Methanol extracts of the skin of the rare South African leptodactylid frog *Heleophryne purcelli* contain a bradykinin-like peptide. This has been isolated in a pure form and recognized to be Hyp^3-bradykinin:

Arg-Pro-Hyp-Gly-Phe-Ser-Pro-Phe-Arg

Materials. The fresh skins of 14 specimens of *Heleophryne purcelli depressa* and 63 specimens of *Heleophryne purcelli orientalis* collected in South Africa, Cape, during the period 1972–1975, were used in this study. The material weighed 50.8 g (average 0.66 g per fresh skin). The skins were removed from the frogs immediately after killing and extracted twice with a volume of methanol 5 times the weight of the tissue. The methanol extracts were combined and filtered and then stored in the refrigerator.

Isolation procedure. Samples of pure peptide were obtained by submitting the extracts to the following purification steps: a) washing the evaporation residue with petroleum ether in order to eliminate fat contaminants; b) passage through an alumina column which was eluted with ethanol of descending concentrations; c) chromatography on SP-Sephadex column (NH_4^+ form, 6 × 300 mm) which was eluted, in 3-ml fractions, with 120 ml of 0.15 N $HCOONH_4$, pH 6.5, and then with 30 ml of 1 N $HCOONH_4$, pH 6.5. The activity appeared at the top of 1 N ammonium formiate elution (fractions 41–46). Activity was followed by bioassay (guinea-pig ileum and rat uterus preparations). The final yield in pure peptide was approximately 3×10^{-7} moles, i.e. 300–350 µg per g fresh tissue.

Structure. Amino acid composition, after the usual acid hydrolysis, was as follows: Arg 2, Hyp 1, Pro 2, Ser 1, Gly 1 and Phe 2. It may be seen that the composition was exactly the same as that of bradykinin, with the sole exception of a hydroxyproline residue replacing a proline residue. The R_f value of the dansyl derivative of *Heleophryne* bradykinin, as compared to that of bradykinin, is slightly lower in silica gel H TLC, using the solvent system iso-propanol:methyl acetate:28% ammonia (9:7:4).

The dansylated peptide was not attacked by trypsin, but it cleaved following chymotrypsin digestion (20 µg peptide in 50 µl 0.1 triethylamine bicarbonate buffer at pH 8.2 plus 10 µl of a 0.1% chymotrypsin solution) giving origin to 3 dansylated fragments. 2 of them corresponded to DNS-Ser-Pro-Phe- and DNS-Arg, respectively. The 3rd dansylated fragment, which did not correspond to DNS-Arg-Pro-Pro-Gly-Phe, was purified and its amino acid composition determined in an amino acid analyser. The fragment appeared to be DNS-Arg(Pro,Hyp,Gly)Phe. Finally, 40 µg of the pure peptide was analysed by the dansyl Edman procedure with results shown below:

Arg-Pro-Hyp-Gly-Phe-Ser-Pro-Phe-Arg

Hyp^3-bradykinin was present in all examined species and subspecies of *Heleophryne.* However, whereas in methanol extracts of different batches of *H. purcelli purcelli, H. purcelli depressa* and *H. purcelli orientalis* it varied between 200 and 500 µg/g (expressed as bradykinin, rat uterus preparation) in extracts of *H. natalensis* it was consistently lower, barely attaining 15–25 µg/g fresh skin.

Crude extracts of *H. purcelli depressa* skin contained 150 µg 5-HT base/g tissue; extracts of *H. purcelli orientalis* 360 µg/g. The amine was separated from Hyp^3-bradykinin already during passage of the crude extract through alumina column.

It is worth remembering that the nonapeptide Hyp^3-bradykinin is included in the molecule of Vespakinin-M, Gly-(Hyp^3-bradykinin)-Ile-Asp, isolated by Kishimura et al.[2] from the venom apparatus of *Vespa mandarina.*

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