

## Biosynthesis of Animal and Plant Bufadienolides. Parallel Experiments with Pregn-5-en-3- $\beta$ -ol-20-one-20-<sup>14</sup>C in *Scilla maritima* and *Bufo paracnemis*

It has been shown that pregnenolone plays a very important part in the biosynthesis of cardenolides and related compounds<sup>1</sup>. In experiments with *Helleborus atrorubens*, TSCHESCHE et al.<sup>2</sup> have found that pregnenolone-21-<sup>14</sup>C was a good precursor of the bufadienolide hellebrin, but as far as we know no experimental work has been done on the biosynthesis of bufadienolides from animals using this steroid. Previous experiments on the biosynthesis of marinobufagin have shown<sup>3</sup> that cholesterol was a precursor of the bufadienolide in the toad *B. marinus*, but considering that the label was on carbon-4 and the general observation that the steroid nucleus is a relative stable moiety while the side chain is extensively cleaved during its metabolism, this result did not clarify the biosynthetic problem of the  $\alpha$ -pyrone ring of marinobufagin.

Continuing with our work on the biosynthesis of toad venoms<sup>4</sup> several intact specimens of the toad *Bufo paracnemis* Lutz 1925 were injected s.c. with pregnenolone-20-<sup>14</sup>C<sup>5</sup> and 33 and 80 days after injection the venom from the parotid and tibial glands was collected by simple pressure. Marinobufagin was isolated by chromatographic procedures already described<sup>6</sup>.

At the same time, trying to find a correlation between bufadienolides from both origins, 2 bulbs of *Scilla maritima* (red squill) were inoculated with the same labelled steroid. The plants were harvested 14 days after injection and the bufadienolide scilliroside was isolated by known methods<sup>7</sup>. The results are shown in the Table.

Tracer experiments with pregnenolone-20-<sup>14</sup>C in *Scilla maritima* and in *Bufo paracnemis*

	Precursor specific activity (dpm/mM) <sup>a</sup>	Bufadienolide isolated	Specific activity (dpm/mM)	Specific incorporation (%)
<i>Scilla maritima</i>	$3.9 \times 10^8$	Scilliroside	$2.2 \times 10^6$	0.56
<i>Bufo paracnemis</i>	$7.3 \times 10^8$	Marinobufagin	$6.5 \times 10^{2b}$ $6.8 \times 10^{2c}$	0.0001 0.0001

<sup>a</sup> Radioactivities were determined with a Packard Tri-Carb Model 3305 liquid scintillation spectrometer in the usual scintillation solutions. <sup>b</sup> 33 days' collection. <sup>c</sup> 80 days' collection.

The results clearly indicate that, as expected, pregnenolone was a good precursor of the plant bufadienolide scilliroside, but it was incorporated into the animal bufadienolide marinobufagin in such a value that could be neglected.

Accordingly, it is possible to postulate that, in the case of toads, cholesterol would be first converted into an intermediate with a bile acid structure<sup>8</sup> which could be subsequently transformed into the bufadienolide by unexceptional steps; the isolation from toad venom<sup>9</sup> of 7- $\alpha$ -hydroxy and 7- $\beta$ -hydroxy-cholesterol, a critical intermediate in the biosynthesis of bile acids<sup>10</sup>, would support this hypothesis although no 7-hydroxy-bufadienolide has been detected yet. In the case that cholesterol were metabolized to pregnenolone, this would not be used by the animal to synthesize bufadienolides<sup>11</sup>.

**Zusammenfassung.** Pregnenolon-20-<sup>14</sup>C wird von roten Meerzwiebeln als Vorstufe für herzwirksame Glykoside verwendet, dagegen kann *Bufo paracnemis* diese Produkte, subkutan appliziert, nicht in die üblichen Krötengifte überführen.

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<sup>11</sup> We thank the Consejo Nacional de Investigaciones Científicas y Técnicas (Buenos Aires) and FORGE (New York) for financial support.

## Synthetic Substitute Enzymes: Part III Glutamic Acid Copolymers with Lysozyme-like Activity<sup>1</sup>

Significant lysozyme-like activity was reported by us for copolymers of Glu and Phe, and of Glu and  $\gamma$ -cholesteryl-L-glutamate<sup>2</sup>. These polymers were synthesized in view of the formulation of a mechanism for the  $\beta$ -(1-4)-glucosaminidase activity of lysozyme<sup>3</sup>. The most important feature of this mechanism, the protonation of the glycosidic oxygen atom at the point of fission of the polysaccharide chain by the unionized  $\gamma$ -carboxyl of Glu residue 35, and the stabilization of the carbonium ion formed after bond fission by the carboxylate ion of Asp residue 52, are made possible since these 2 amino-acid residues are located in hydrophobic and hydrophilic re-

gions of the enzyme molecule respectively. Synthesis of Glu copolymers with Phe and  $\gamma$ -cholesteryl-L-glutamate were, therefore, carried out from the N-carboxyanhydrides of  $\gamma$ -benzyl-L-glutamate and the hydrophobic

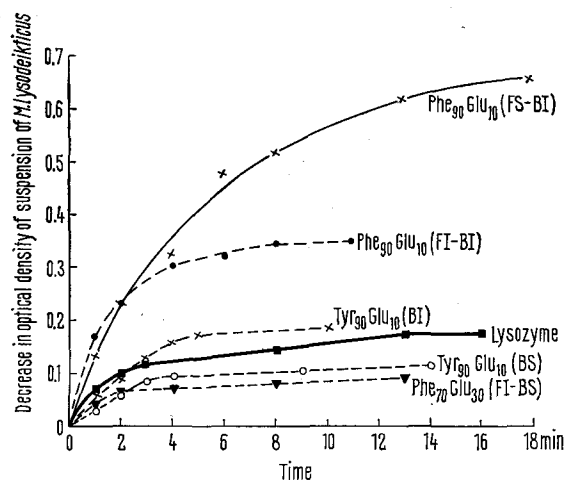
<sup>1</sup> Communication No. 1415 from the Central Drug Research Institute. Part II – V. K. NAIETHANI, K. B. MATHUR and M. M. DHAR, Indian J. Biochem. 6, 10 (1969).

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amino-acid in 2 stages. In the first the N-carboxyanhydride of the hydrophobic amino-acid was in excess, and in the second the ratio was reversed. Subsequent removal of the benzyl groups yielded peptides having carboxyl functions in both hydrophobic and hydrophilic regions. Amino-acid ratios of the active fractions from these copolymerizations, however, revealed that activity was only observable in peptides having an excess of the hydrophobic amino-acid. A random copolymer of Glu and a hydrophobic amino-acid would be expected to have Glu residues and, therefore, carboxylic functions in the inner hydrophobic region as well as the outer surface, which would be in a hydrophilic environment. Copolymers of Phe-Glu, Tyr-Glu and Ala-Glu have, therefore, been synthesized containing different ratios of the 2 amino-acids. Some of these copolymers, indeed, have very substantial lysozyme-like activity (Figure).

For the synthesis of these Glu copolymers, the N-carboxyanhydrides of  $\gamma$ -benzyl-L-glutamate and the second amino-acid in the appropriate ratio were dissolved in dioxane (1 mmol/100 ml) and polymerization initiated with triethylamine (0.005 mmol/100 ml). Deblocking of



Lytic activity of synthetic Glu copolymers (0.5 mg/ml) and hen egg-white lysozyme (0.05 mg/ml). FI, formic acid insoluble; FS, formic acid soluble; BI, bicarbonate insoluble; BS, bicarbonate soluble.

the precipitated copolymers was achieved by treatment with 2 N HBr in AcOH. The Phe-Glu copolymers were separated into formic-acid soluble and insoluble fractions before treatment with HBr, and all copolymers were fractionated into bicarbonate soluble and insoluble materials.

Lytic activity of the copolymers was assessed by their ability to decrease the turbidity of suspensions of *Micrococcus lysodeikticus* as described earlier<sup>2</sup>. The active copolymers also degraded the cell wall of *M. lysodeikticus* with liberation of reducing sugars. The most active polymer, Phe<sub>90</sub>Glu<sub>10</sub><sup>4</sup> and egg-white lysozyme degraded the cell wall of *M. lysodeikticus* to glycopeptides that were indistinguishable on paper electrophoresis (0.1 M sodium borate-HCl buffer, pH 6.5; 3.8 volts/cm for 18 h). Paper electrophoresis of 6 N HCl hydrolysates of these glycopeptides revealed the same ninhydrin-positive zones. Phe<sub>90</sub>Glu<sub>10</sub> has also been found to make amoebic cysts susceptible to emetine hydrochloride in a manner analogous to egg-white lysozyme<sup>5</sup>.

It is not possible to get an absolute assessment of the activity of Phe<sub>90</sub>Glu<sub>10</sub> as this polymer is insoluble. Assays have, therefore, been carried out with suspensions. Even so, it would appear (Figure) that Phe<sub>90</sub>Glu<sub>10</sub> has about 1/3 the activity of egg-white lysozyme and the bicarbonate-soluble Phe<sub>70</sub>Glu<sub>30</sub> one-tenth the activity. Tyr<sub>90</sub>Glu<sub>10</sub> is far less active than the corresponding Phe-Glu copolymer Phe<sub>90</sub>Glu<sub>10</sub>. Copolymers Ala<sub>90</sub>Glu<sub>10</sub>, Ala<sub>70</sub>Glu<sub>30</sub>, Ala<sub>50</sub>Glu<sub>50</sub>, Tyr<sub>70</sub>Glu<sub>30</sub>, Tyr<sub>50</sub>Glu<sub>50</sub> and Phe<sub>50</sub>Glu<sub>50</sub> are all inactive.

**Zusammenfassung.** Synthetische Copolymere aus Glutaminsäure und Phenylalanin zeigen Lysozymaktivität. Mit einem Produkt Phe<sub>90</sub>Glu<sub>10</sub> wurde ca. ein Drittel der Lysozymaktivität erreicht.

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<sup>4</sup> Copolymers designated according to molar percentage of N-carboxyanhydrides used for their synthesis. Actual amino-acid analysis correspond to these values closely.

<sup>5</sup> S. A. IMAM, unpublished data.

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## Pendulin, a New Biscoclaurine Alkaloid from *Cocculus pendulus* Diels<sup>1</sup>

During a programme for screening plant extracts for biological activities, hypotensive and anticancer activities were seen in the 50% ethanol extract of the leaves and stem of *Cocculus pendulus* (Forsk) Diels (Syn. *C. leaeba* DC)<sup>2</sup>. Follow-up studies located both these activities in the alkaloidal fraction, which, as a result, has been taken up for more detailed studies.

Columbine and palmitine have been found earlier in this plant<sup>3</sup>. More recently coclaurine, menisarine and sinactine have also been located<sup>4</sup>.

In the present investigation<sup>5</sup>, a new biscoclaurine base, designated pendulin, (C<sub>27</sub>H<sub>40</sub>N<sub>2</sub>O<sub>6</sub>) (M<sup>+</sup>, 608), mp 192–194°, [α]<sub>D</sub><sup>25</sup> + 265° has been isolated. Pendulin forms a hydrochloride mp 276–278°, a picrate mp 210–212° and a dimethiodide mp 282–286°. Its IR-spectrum in KBr has absorption bands at 3322, 2857, 1587, 1506, 1458, 1372, 1267, 1221, 1117, 1070, 1020 and 972 cm<sup>-1</sup> indicating

the presence of hydroxyl and ether functions in the molecule and its aromatic nature. Its UV maximum at 284 nm (log ε, 3.84) in ethanol is bathochromically shifted to 306 nm on addition of sodium hydroxide in a manner typical for bis(benzyl)isoquinoline bases<sup>6</sup>.

<sup>1</sup> Communication No. 1414 from the Central Drug Research Institute.

<sup>2</sup> D. S. BHAKUNI, M. L. DHAR, M. M. DHAR, B. N. DHAWAN and B. N. MEHROTRA, Indian J. exp. Biol., in press.

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<sup>5</sup> Satisfactory C, H and N analysis of all reported compounds obtained and optical rotations routinely determined in CHCl<sub>3</sub>. UV-spectra in EtOH, IR-spectra in KBr and 60 Mcs NMR-spectra in CDCl<sub>3</sub> with TMS as an internal standard.

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