Glu(OMe)^2-litorin, the second bombesin-like peptide occurring in methanol extracts of the skin of the Australian frog *Litoria aurea*[1]

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Summary. The second bombesin-like peptide occurring in methanol extracts of the skin of the Australian frog Litoria aurea was isolated in a pure form and identified as Glu(OMe)^2-litorin.

Methanol extracts of the skin of the Australian frog Litoria (Hyla) aurea were found to contain 2 peptides possessing bombesin-like activity. The structure of one of them has been described in a preceding communication[2]. In this paper the structure of the second bombesin-like peptide will be described. This differs from that of the first peptide only by the replacement of the glutamine residue present in the litorin sequence at position 2 with the γ-methylester of glutamic acid.

Pyr-Gln—Trp-Ala-Val-Gly-His-Phe-Met-NH_2 Litorin
Pyr-Glu(OMe)-Trp-Ala-Val-Gly-His-Phe-Met-NH_2
Glu(OMe)^2-litorina

Materials. The dried skins of 1018 specimens of L. aurea collected near Melbourne, Victoria, Australia, in October–December 1973 and weighing a total of 610 g, were subjected to 2 successive extractions with 20 parts (w/v) of 80% methanol, each extraction lasting 1 week. The extracts were mixed and filtered.

Isolation procedure. Almost the whole extract, corresponding to 610 g of dried skin, was evaporated to dryness. The residue was washed with petroleum ether and then taken up in water plus 99% ethanol to give a final ethanol concentration of 95% (1200 ml). After standing, the limpid supernatant was passed through 12 columns of alkaline alumina, each of 170 g, which were then eluted with ethanol-water mixtures of decreasing concentrations of ethanol, each of 200 ml.

2 peaks of bombesin-like activity appeared in the ethanol eluates, one in eluates $95_2 + 95_3 + (90_1)$ and the other in eluates $90_2 + (90_3)$. Litorin was responsible for the second peak of activity; Glu(OMe)^2-litorin for the first peak. In order to obtain a sharper separation of Glu(OMe)^2-litorin from litorin, eluates 95_2, 95_3 and 90_1 were combined, evaporated to dryness and the residue taken up with 600 ml of 99% ethanol. The liquid was then re-chromatographed on 6 alumina columns and elution was carried out as above.

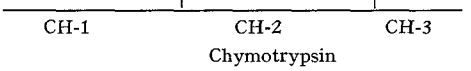
In this situation, Glu(OMe)^2-litorin emerged again, together with some bufotenidine, in eluates $95_2 + 95_3 + 95_4$ and was completely free of litorin, which emerged in eluates $90_2 + 80$. Purification of eluates $95_3 + 95_4$ (containing approximately 30 µg polypeptide per g skin, as assessed on the rat uterus preparation) was carried out by gel filtration on Sephadex G-10 followed by preparative paper electrophoresis.

Litorin and Glu(OMe)^2-litorin were found to possess the same electrophoretic mobility in acid and neutral media, and the amino acid composition of their total acid hydrolysate was found to be identical. However, a difference in their structures was demonstrated by thin layer chromatography on silica gel, in which the 2 peptides had the following R_f values:

	Litorin I	Glu(OMe)^2-Litorin
1. n-Butanol-acetic acid:water (4:1:1)	0.35	0.45
2. n-Butanol:pyridine:acetic acid:water (4:1:1:1)	0.55	0.65
3. n-Butanol:diethylamine:water (4:1:1)	0.15	0.3

Structure. Chymotryptic digestion of litorin and Glu(OMe)^2-litorin hydrolyzed the Trp and Phe bonds producing three fragments in each case. The CH-2 and CH-3 fragments of the 2 peptides were identical, possessing the same amino acid content and exhibiting identical electrophoretic and chromatographic behaviour.

Pyr-Gln—Trp-Ala-Val-Gly-His-Phe-Met-NH_2
Pyr-Glu(OMet)-Trp-Ala-Val-Gly-His-Phe-Met-NH_2



Chymotrypsin

The CH-1 fragments of both peptides contained 2 glutamyl and one tryptophanyl residues and they had the same electrophoretic mobility. However, they showed remarkable differences in their R_f values on thin layer chromatography, as shown below:

	Litorin	Glu(OMe)^2-litorin
2.	0.38	0.56
3.	0.2	0.35

Similar observations were made for the dipeptide fragments obtained after removal of Trp from CH-1 with carboxypeptidase A.

	Litorin	Glu(OMe)^2-litorin
2.	0.6	1.2
3.	0.51	1.1

The above observations made it clear that the Glu residue in the second position from the N-terminus had to be present in Glu(OMe)^2-litorin in a form different from the amide and was probably present in the γ-ester form.

This hypothesis was confirmed by comparing the R_f values of the dipeptide Pyr-Glu(OMe) and of the tripeptide Pyr-Glu(OMe)-Trp prepared by synthesis, with the corresponding fragment obtained from Glu(OMe)^2-litorin: they were found to be identical in all the above systems. The exactness of the structure proposed has been confirmed by the perfect superimposition of the biological spectra of natural Glu(OMe)^2-litorin and Glu(OMe)^2-litorin prepared by synthesis[3]. As far as we know, this is the first time that a Glu residue has been found in the γ-ester form in a peptide isolated from materials of natural origin. Experiments are in progress to solve the problem of whether Glu(OMe)^2-litorin pre-exists as such in tissues or is an artifact stemming from the use of methanol in the extraction of the skins.

Glu(OMe)^2-litorin possesses a spectrum of biological activity similar to that of litorin. However, the 2 peptides may easily be distinguished from each other by parallel bioassay.

1 Supported in part by grants from the Consiglio Nazionale delle Ricerche, Roma.

2 A. Anastasi, V. Erspamer and R. Endean, Experientia *31*, 510 (1975).

3 M. Mazzoli and R. de Castiglione, Experientia *33*, 990 (1977).