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Isolation of L-3-phenyllactyl-Leu-Arg-Asn-NH_2 (Antho-RNamide), a sea anemone neuropeptide containing an unusual amino-terminal blocking group

(coelenterate/neurotransmitter/peptide hormone/posttranslational modification/mass spectrometry)

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ABSTRACT **Using a radioimmunoassay for the carboxyl-terminal sequence Arg-Asn-NH_2, we have purified a peptide from acetic acid extracts of the sea anemone *Anthopleura elegantissima*. By classical amino acid analyses, mass spectrometry, and ^1H NMR spectroscopy, the structure of this peptide was determined as 3-phenyllactyl-Leu-Arg-Asn-NH_2. By using reversed-phase HPLC and a chiral mobile phase, it was shown that the 3-phenyllactyl group had the L configuration. Immunocytochemical staining with antiserum against Arg-Asn-NH_2 showed that L-3-phenyllactyl-Leu-Arg-Asn-NH_2 (Antho-RNamide) was localized in neurons of sea anemones. The L-3-phenyllactyl group has not been found earlier in neuropeptides of vertebrates or higher invertebrates. We propose that this residue renders Antho-RNamide resistant to nonspecific aminopeptidases, thereby increasing the stability of the peptide after neuronal release.**

Coelenterates were probably the first group of animals that evolved a nervous system. Electrophysiological and ultrastructural investigations have shown that coelenterate neurons use transmitters or locally acting hormones for signal transmission (1–5). Until recently, however, the nature of such transmitter substances has remained unknown. By means of immunocytochemistry with antisera to the sequence Arg-Phe-NH_2 (RFamide), RFamide-like peptides were demonstrated in the nervous systems of animals belonging to all classes of coelenterates (5–9). With a radioimmunoassay for RFamide, three RFamide-like peptides were isolated from the sea anemone *Anthopleura elegantissima* and sequenced: <Glu-Gly-Arg-Phe-NH_2 (Antho-RFamide), <Glu-Ser-Leu-Arg-Trp-NH_2 (Antho-RWamide I), and <Glu-Gly-Leu-Arg-Trp-NH_2 (Antho-RWamide II) (10–12). The general structure of these peptides can be described as <Glu . . . Arg-Xaa-NH_2, where Xaa is an aromatic amino acid. From the hydromedusa *Polyorchis penicillatus* the peptide <Glu-Leu-Leu-Gly-Gly-Arg-Phe-NH_2 (Pol-RFamide I) was isolated, which also belongs to the <Glu . . . Arg-Xaa-NH_2 family (13). Use of specific antisera showed that all isolated coelenterate peptides were located in neurons, most of which were associated with smooth muscle fibers (5, 11, 13). Application of low doses of Antho-RFamide or Antho-RWamide I or II induced contractions of endodermal muscles of sea anemones (5, 14). This strongly suggests that these peptides are involved in neurotransmission.

The neuropeptides from coelenterates have many features in common with neuropeptides that are produced by higher animals: They are probably synthesized as a high molecular weight precursor (5), stored in dense-cored vesicles, and released by exocytosis (15). Like many other neuropeptides, the coelenterate peptides have an amidated carboxyl terminus and an amino-terminal pyroglutamyl group. These groups, in addition to having other functions (e.g., in receptor binding), also protect the peptide against degradation by nonspecific carboxy- and aminopeptidases. In the present paper we describe the isolation of a neuropeptide from sea anemones that contains a phenyllactyl group at its amino terminus. As this amino terminus does not have a free amino group, it might represent another way in which nature protects biologically active peptides against nonspecific enzymatic degradation.

METHODS

Radioimmunoassays. The RFamide radioimmunoassay was described earlier (10). For the Arg-Asn-NH_2 (RNamide) radioimmunoassay, four rabbits were immunized with RNamide that was coupled to bovine thyroglobulin (Sigma) via carbodiimide. The antisera were treated as described (10). Antiserum 197, bleeding VII (197 VII) was selected for the radioimmunoassay. It was used in a dilution of 1:10,000. ^{125}I-labeled Tyr-Arg-Asn-NH_2 (YRNamide) was prepared by the method described in ref. 10, except that instead of methanol, increasing concentrations of acetonitrile in 0.1% trifluoroacetic acid (TFA) were used for elution of the tracer. All further information concerning the radioimmunoassay is given in ref. 10.

Immunochemistry. *Calliactis parasitica* were obtained from The Marine Laboratory (Plymouth, U.K.). They were fixed and rinsed as described (6–8), embedded in Tissue-Tek (Miles), frozen, and sectioned by a cryostat microtome. Antiserum 219 II, directed against RNamide (see above), was used in a dilution of 1:200. Staining was as in ref. 8.

Sea Anemone Extract. *A. elegantissima* were purchased from Biomarine Laboratories (Venice, CA). Frozen tissue was minced and boiled for 30 min in twice-distilled water (2 ml/g wet weight). After cooling to 0°C, the mixture was brought to 0.1 M acetic acid and adjusted with dilute HCl to pH 3. This was homogenized with a kitchen mixer and centrifuged at 5000 × *g* for 15 min. The pellets were reextracted with 0.1 M acetic acid (pH 3) (1 ml/g wet weight) and the combined supernatants were brought to pH 7 with ammonia. The extract was subsequently pressed through methanol/water-pretreated Sep-Pak C_{18} cartridges (Waters; 40 ml of extract per cartridge). All cartridges were washed with twice-distilled water (10 ml per cartridge) and eluted

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Abbreviations: RFamide, Arg-Phe-NH_2; RNamide, Arg-Asn-NH_2; YRNamide, Tyr-Arg-Asn-NH_2; FABMS, fast-atom bombardment mass spectrometry; CID, collisionally induced decomposition; TFA, trifluoroacetic acid.

with 100% methanol (20 ml per cartridge). The methanol and part of the water were removed by rotary evaporation, and the remaining aqueous solution was brought to 0.3 M ammonium acetate (pH 7) and further purified by cation-exchange chromatography.

Chromatography. For cation-exchange chromatography, CM-Sephadex C-25 (Pharmacia) was used. Columns (Pharmacia) were connected to a Multirac/Uvicord II chromatography apparatus (LKB). HPLC was carried out with a Shimadzu LC-6A system, except for the separation of the two stereoisomers of phenyllactate, where a Beckman HPLC (system gold, delivery module 126, UV detector 166) was used. The HPLC columns containing Spherisorb ODS-2 or Partisil ODS-3 were purchased from Latek (Heidelberg), and those containing Nucleosil C_{18} and Nucleosil C_8 from Macherey & Nagel (Düren, F.R.G.).

Peptide Chemistry. Amino acid composition was determined after overnight hydrolysis of the peptide in 6 M HCl at 115°C, followed by dansylation (volume, 20 μl) and subsequent separation of the dansylated amino acids on F1700 polyamide sheets (2.5 × 2.5 cm; Schleicher & Schüll) (16–18). End-group determination was carried out with both the dansyl chloride and the 4-(*N,N*-dimethylaminoazo)benzene-4′-isothiocyanate method (16–19). Digestion of intact peptide with trypsin was carried out as described (11). After this digestion, the liberated amino acid amides were dansylated and identified by chromatography on polyamide sheets (20). Acetylation was carried out by treating peptide with excess acetic anhydride and triethylamine at room temperature overnight, then removing excess anhydride and amine *in vacuo*. The crude material was employed for spectroscopy. Arg-Asn-NH_2, Arg-Gly-NH_2, Arg-His-NH_2, Arg-Leu-NH_2, Arg-Met-NH_2, Arg-Pro-NH_2, Arg-Ser-NH_2, Arg-Thr-NH_2, Arg-Trp-NH_2, Arg-Val-NH_2, <Glu-Gly-Arg-Phe-NH_2, L-3-phenyllactyl-Leu-Arg-Asn-NH_2, Tyr-Arg-Asn-NH_2, and D-3-phenyllactic acid were from custom syntheses carried out by Bachem (Bubendorf, Switzerland). Neuromedin U-8 was from Bachem; DL-3-phenyllactic acid was from Sigma; L-3-phenyllactic acid and DL-tropic acid were from Aldrich.

Spectroscopy. Mass spectra were obtained on either a ZAB-SE or a 70-SE4F (VG Analytical, Manchester, U.K.) mass spectrometer operating at 8-kV accelerating potential. Ionization was by the fast-atom bombardment (FAB) method, employing an Ion Tech ion gun and a xenon beam at ≈8 keV. Tandem FAB mass spectrometry (FABMS/MS) and high-resolution measurements were made on the 70-SE4F. ^1H NMR spectra were obtained on a GN500 (General Electric) spectrometer in ^2H_2O solution with solvent presaturation, employing dioxane (δ 3.53) as internal standard.

RESULTS

Radioimmunoassay for RNamide Peptides. During earlier purifications of Antho-RFamide and the Antho-RWamides I and II from extracts of *A. elegantissima*, we discovered other components that were weakly immunoreactive in an RFamide radioimmunoassay (11, 12, 21). One of these components was further purified and its amino acid composition determined as Leu, Arg, and Asx (21). Trypsin treatment of this peptide yielded Asn-NH_2, showing that the carboxyl terminus had to be Arg-Asn-NH_2 (21). To obtain enough of this Arg-Asn-NH_2 (RNamide) peptide for further structure analyses, we started a large-scale purification (see below). To better monitor the isolation of the RNamide peptide, we developed an RNamide radioimmunoassay (Fig. 1). This radioimmunoassay recognizes free RNamide and elongated peptides containing the carboxyl-terminal sequence RNamide. The sensitivity of the radioimmunoassay for elongated RNamide peptides is high (50% displacement at ≈2 × 10^{-13} mol of Tyr-Arg-Asn-NH_2). The assay is also rather specific (Fig. 1).

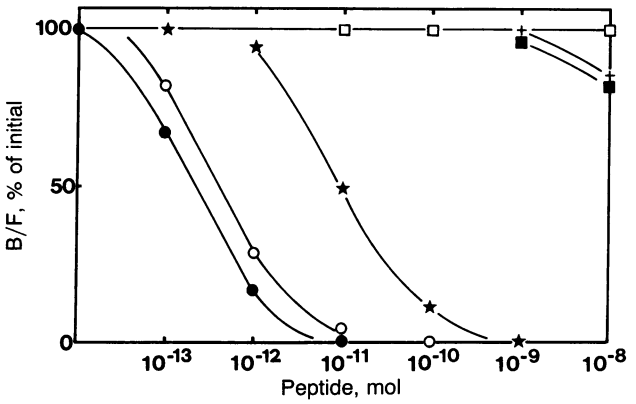

FIG. 1. Specificity of the RNamide radioimmunoassay. B/F, bound/free tracer. ●, Tyr-Arg-Asn-NH_2; ○, neuromedin U-8 (Tyr-Phe-Leu-Phe-Arg-Pro-Arg-Asn-NH_2); ★, Arg-Asn-NH_2; ■, Arg-Ser-NH_2, <Glu-Gly-Arg-Phe-NH_2; +, Arg-Val-NH_2; □, Arg-Gly-NH_2, Arg-His-NH_2, Arg-Leu-NH_2, Arg-Met-NH_2, Arg-Pro-NH_2, Arg-Thr-NH_2, Arg-Trp-NH_2, <Glu-Gly-Leu-Arg-Trp-NH_2.

Purification of the RNamide Peptide. In an acetic acid extract of 4.8 kg (wet weight) of *A. elegantissima* we measured 700 nmol of Tyr-Arg-Asn-NH_2 (YRNamide) equivalents. Of this, 500 nmol could be bound to Sep-Pak C_{18} cartridges. Rinsing of the C_{18} cartridges with twice-distilled water removed 40 nmol of YRNamide equivalents. Subsequent elution with 100% methanol yielded 300 nmol of immunoreactive material, which was considerably purified and desalted. This material was further purified by cation-exchange chromatography (Fig. 2). Two immunoreactive fractions appeared: one large fraction that was eluted at 0.7–1.6 M and a second, smaller fraction appearing at 2.4–2.7 M ammonium acetate (Fig. 2). The first fraction, which contained most of the immunoreactivity (210 nmol of YRNamide equivalents), was lyophilized and subsequently purified by reversed-phase HPLC (Fig. 3 *a–c*). After this, the immunoreactive material was pure (Fig. 3*d*).

Structure of the Peptide. An aliquot (1 nmol) of the purified material was hydrolyzed and its amino acid composition determined. In agreement with our previous findings (see above), we found Leu, Arg, and Asx, with no other amino acids visible on our polyamide chromatograms. Digestion of

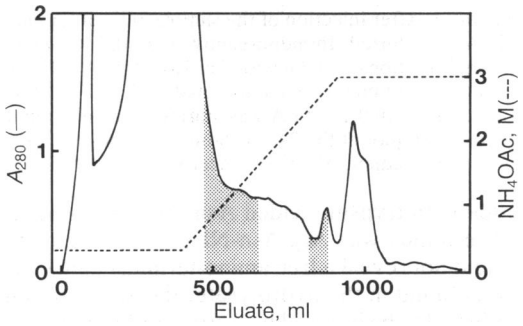

FIG. 2. Cation-exchange chromatography of an acetic acid extract of 4.8 kg of *A. elegantissima*. This extract was desalted as described in the text. The column (2.6 × 56 cm; void volume, 110 ml) contained CM-Sephadex C-25 equilibrated with 0.3 M NH_4OAc (pH 7). After application of the sample (145 ml), the column was washed with 240 ml of buffer and then a linear gradient of 0.3–3.0 M NH_4OAc (pH 7) was started (20 hr, 24 ml/hr). The column was subsequently washed with 400 ml 3.0 M NH_4OAc (pH 7). RNamide-immunoreactive fractions (stippled) appeared at 0.7–1.6 M and 2.4–2.7 M NH_4OAc (210 nmol and 90 nmol of YRNamide equivalents, respectively).

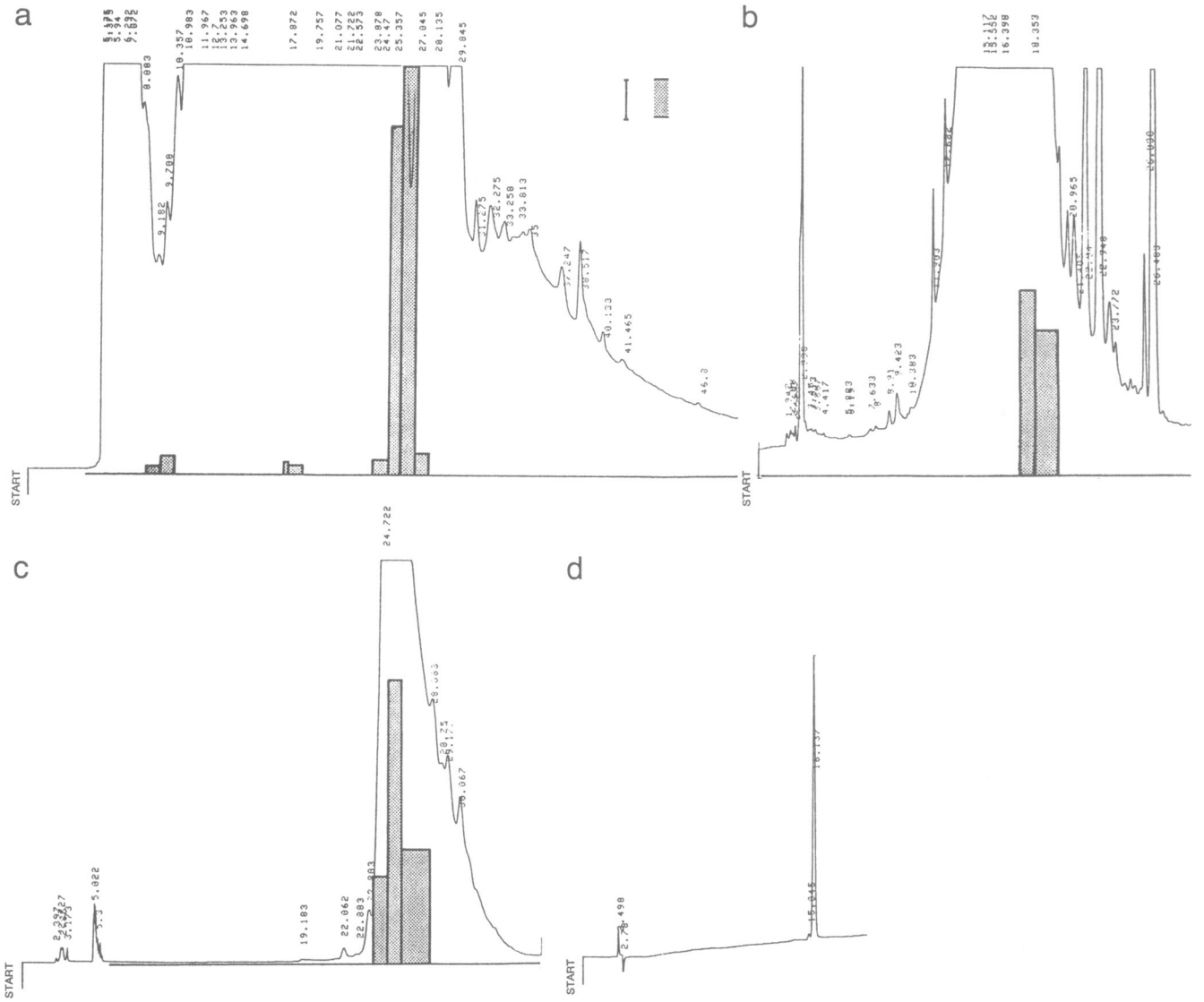

FIG. 3. Purification of the RNamide-immunoreactive peptide by HPLC. Vertical bar, 2.0 ΔA at 215 nm; vertical stippled area, 4 nmol of YRNamide equivalents. (*a*) Preparative HPLC of the immunoreactive fraction appearing at 0.7–1.6 M NH_4OAc in Fig. 2 (after lyophilization). Of a total of 180 nmol of YRNamide equivalents (2.5 ml), 68 nmol (950 μl) was injected. The column (Spherisorb ODS-2; particle size, 5 μm; pore diameter, 80 Å; dimensions, 20 × 250 mm) was equilibrated with 10% acetonitrile in 0.1% TFA (8 ml/min). One minute after injection of the sample, a linear gradient of 10–60% acetonitrile in 0.1% TFA was started (40 min). A major immunoreactive fraction was eluted at 24–28 min (60 nmol of YRNamide equivalents). (*b*) HPLC of the combined 24- to 28-min fractions of *a*. Of a total of 137 nmol of YRNamide equivalents (380 μl), 34 nmol (95 μl) was injected. The column (Nucleosil C_{18}, 5 μm, 120 Å, 4 × 250 mm) was equilibrated with 10% acetonitrile in 0.1% TFA (1 ml/min). After injection of the sample, a linear gradient of 10–22% (10 min) followed by a linear gradient of 22–35% (20 min) acetonitrile in 0.1% TFA was started. Immunoreactive material was eluted at 18–20.7 min (30 nmol of YRNamide equivalents). (*c*) HPLC of the combined 18- to 20.7-min fractions of *b*. Of a total of 124 nmol of YRNamide equivalents (250 μl), 47 nmol (95 μl) was injected. The column (Partisil ODS-3, 5 μm, 60 Å, 4 × 250 mm) was equilibrated with 10% acetonitrile in 0.1% TFA (1 ml/min). After injection of the sample, a linear gradient of 10–27% acetonitrile in 0.1% TFA was started (30 min). Immunoreactive material was eluted at 24–28 min (46 nmol of YRNamide equivalents). (*d*) HPLC of an aliquot of the 24- to 28-min fraction purified in *c* to show that this material was >98% pure (column of Nucleosil C_8, 5 μm, 120 Å, 4 × 250 mm; gradient of 10–35% acetonitrile in 0.1% TFA, 1 ml/min, 20 min).

the peptide with trypsin yielded Asn-NH_2, showing that the carboxyl terminus was Arg-Asn-NH_2. An end-group determination did not reveal a free amino-terminal amino acid. As no Glu was found in the hydrolysate, the amino terminus of the peptide had to be blocked by a group different from <Glu. For these reasons, classical amino acid sequencing could not be performed and the peptide was, therefore, further analyzed by MS. FABMS yielded an $(M + H)^+$ ion at m/z 549.3135 ($C_{25}H_{41}N_8O_6$, Δ 1.4 mmu), which on study by collisionally induced decomposition (CID) FABMS/MS gave a fragmentation pattern characteristic for the sequence Leu-Arg-Asn-NH_2 (Fig. 4). The amino terminus of the peptide appeared to be an unknown group that did not represent a common amino acid and that had a molecular weight of 149.0589, corresponding to $C_9H_9O_2$ (Δ 1.4 mmu). Upon acetylation of the peptide with acetic anhydride, the molecular weight increased to 590, and in FABMS/CID/MS, the amino-terminal unit yielded an ion at m/z 191, suggesting that it contained a hydroxyl group. Moreover, an ion at m/z 163 (at m/z 121 in the natural peptide) indicated a carbonyl group as well.

^1H NMR spectroscopy of the natural peptide indicated a phenyl group (δ 7.14 d, J = 7.2 Hz, 2H, ortho; δ 7.27 t, J = 6.8 Hz, 2H, meta; δ 7.21 t, J = 7.1 Hz, 1H, para) and a methylene group (CH_2, ABX pattern: δ 2.78 dd, J = 14.5, 7.0 Hz; δ 2.96 dd, J = 14.5, 5.0 Hz) adjacent to an oxymethine unit (carbinol, δ 4.28 t, J = 6.8 Hz). The chemical shifts and coupling patterns were compared to those of model compounds, most notably phenyllactic acid and phenylhydracrylic acid ethyl ester (but also hydroxyethylbenzoyl and

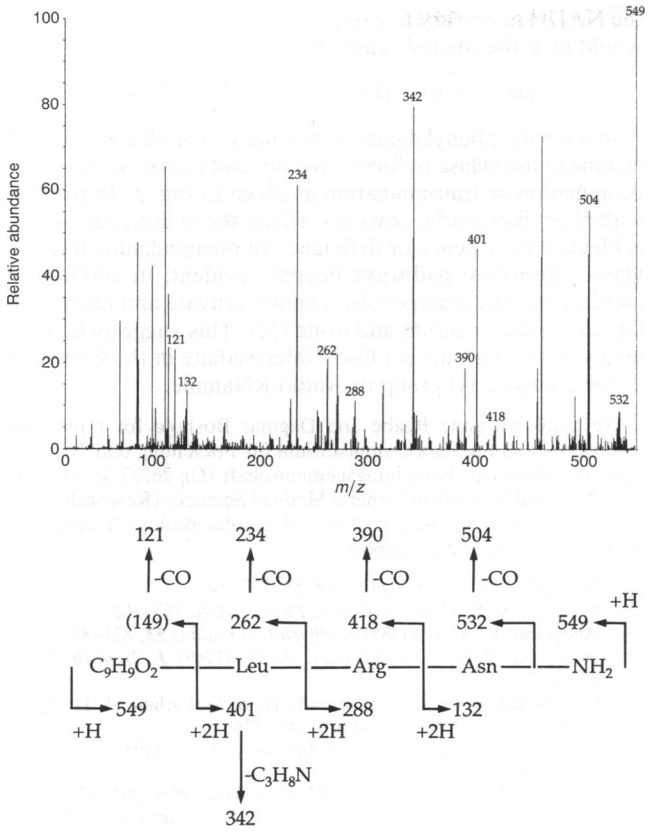

FIG. 4. (*Upper*) FABMS/CID/MS spectrum of the natural peptide at *m/z* 549.3. (*Lower*) Amino acid sequence derived from the fragmentation pattern. The amino terminus is an unknown group with molecular weight 149 corresponding to $C_9H_9O_2$.

phenoxypropionyl derivates), which argued strongly for a 3-phenyllactyl unit at the amino terminus of the peptide.

The presence of a 3-phenyllactyl group in the peptide was also confirmed after HCl hydrolysis and subsequent analysis by reversed-phase HPLC. With a Partisil ODS-3 column (4 × 250 mm), a gradient of 5–21.5% acetonitrile in 0.1% TFA (1.5 ml/min; 15 min) and a monitor wavelength of 254 nm, both synthetic DL-3-phenyllactic acid and the natural compound appeared at 13.8 min (detection limit, 0.5 nmol). Our HPLC analysis was quite selective, as it was possible to separate 3-phenyllactic acid (3-phenyl-2-hydroxypropionic acid) from several closely related organic acids such as tropic acid (2-phenyl-3-hydroxypropionic acid), which under the conditions described above appeared at 12.4 min.

To determine the configuration of the asymmetric C atom (C-2) of the 3-phenyllactyl group, we hydrolyzed 10 nmol of native peptide and purified the 3-phenyllactic acid as described in the preceding paragraph. The natural compound was subsequently injected on an HPLC column (4 × 250 mm) containing Nucleosil C_{18} (pore size, 120 Å) that was equilibrated (flow, 2 ml/min) with 2 mM *N,N*-dimethyl-L-phenylalanine/1 mM copper(II) acetate/10% methanol, pH 4.4 (22). Under these conditions the natural 3-phenyllactic acid was eluted at 29.3 min. Subsequently injected synthetic L-3-phenyllactic acid appeared at the same retention time, whereas synthetic D-3-phenyllactic acid was eluted at 23.1 min, showing that the natural compound was identical to the L-enantiomer.

Table 1. HPLC behavior of natural, purified *Anthopleura* peptide and synthetic L-3-phenyllactyl-L-Leu-L-Arg-L-Asn-NH_2

Column material (pore size)	Acetonitrile gradient (20 min) in 0.1% TFA	Retention time, min: Natural peptide	Retention time, min: Synthetic peptide
Spherisorb ODS-2 (80 Å)	10–35%	19.153	19.140
Nucleosil C_{18} (120 Å)	10–30%	18.830	18.842
Nucleosil C_{18} (300 Å)	10–35%	15.117	15.120
Nucleosil C_8 (120 Å)	10–35%	16.137	16.137
Partisil ODS-3 (60 Å)	10–35%	14.895	14.898

Dimensions of columns were 4 × 250 mm; particle size was 5 μm. Retention-time variability of the HPLC system was ≤1%.

Comparison of Synthetic and Natural Peptide. FABMS/CID/MS on *m/z* 549.3 of synthetic L-3-phenyllactyl-L-Leu-L-Arg-L-Asn-NH_2 gave a fragmentation pattern that was identical to that of the natural peptide. The identity of synthetic and natural peptide was also confirmed by HPLC. Using column materials with different ligands or pore diameters, we found that synthetic L-3-phenyllactyl-L-Leu-L-Arg-L-Asn-NH_2 and the natural peptide were always eluted at the same retention times (Table 1).

Localization of the Peptide. Using immunocytochemistry with antiserum against the sequence Arg-Asn-NH_2, we found that only nerve cells and no other cell types were stained in sea anemones. L-3-Phenyllactyl-Leu-Arg-Asn-NH_2, therefore, is a neuropeptide.

DISCUSSION

The neuropeptide L-3-phenyllactyl-Leu-Arg-Asn-NH_2, which we shall now call Antho-RNamide, has many features in common with the other neuropeptides isolated from coelenterates (Table 2): these peptides are small, consist of only neutral or basic amino acids, have a blocked amino and an amidated carboxyl terminus, and terminate with the sequence Arg-Xaa-NH_2, where Xaa is an uncharged aliphatic or aromatic amino acid. The Arg-Xaa-NH_2 family can be subdivided into smaller families (Table 2). Such a family can occur within one species, which is the case for the Antho-RNamide/Antho-RWamide family in the sea anemone *A. elegantissima* and the Pol-RFamides in the hydromedusa *P. penicillatus*. Family relations, however, can also exist between peptides of species belonging to different classes of coelenterates: Antho-RFamide, which occurs in anthozoans, is related to the Pol-RFamides, which occur in hydrozoans. This suggests that the DNA sequences that code for these peptides have evolved from a common ancestral gene.

An amino-terminal L-3-phenyllactyl group has not been identified earlier in neuropeptides or peptide hormones of higher animals. Amino-terminal <Glu occurs in about 50% of

Table 2. Neuropeptide families in coelenterates

Species	Structure	Name	Ref.
A. elegantissima	Phenyllactyl-Leu-Arg-Asn-NH_2	Antho-RNamide	—
A. elegantissima	<Glu-Ser-Leu-Arg-Trp-NH_2	Antho-RWamide I	11
A. elegantissima	<Glu-Gly-Leu-Arg-Trp-NH_2	Antho-RWamide II	12
A. elegantissima	<Glu-Gly-Arg-Phe-NH_2	Antho-RFamide	10
Renilla köllikeri	<Glu-Gly-Arg-Phe-NH_2	Antho-RFamide	23
P. penicillatus	<Glu-Leu-Leu-Gly-Gly-Arg-Phe-NH_2	Pol-RFamide I	13
P. penicillatus	<Glu-(Trp,Leu,Lys)-Gly-Arg-Phe-NH_2	Pol-RFamide II	5

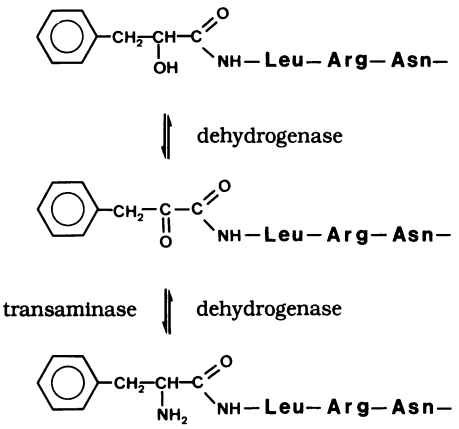

FIG. 5. A possible pathway for the biosynthesis of the L-3-phenyllactyl group (top) from an amino-terminal phenylalanyl residue (bottom).

all known biologically active peptides and protects these substances against breakdown by nonspecific aminopeptidases. Three examples are known where neuroendocrine peptides are "blocked" by an amino-terminal acetyl group (*N*-acetyl-β-endorphin, -α-melanotropin, and -oxytocin) (24–26). The biosynthesis of an amino-terminal L-3-phenyllactyl residue represents another way in which nature protects biologically active peptides.

There are several possibilities for the biosynthesis of the L-3-phenyllactyl group. After a partial processing of the presumed Antho-RNamide precursor protein, phenyllactic acid could be coupled enzymatically to the amino terminus of Leu-Arg-Asn- (catalyzed by a hypothetical enzyme: phenyllactyl-peptide transferase). However, we favor a second possibility, by which a phenylalanyl group would yield a phenyllactyl group after oxidative deamination or transamination and a subsequent reduction step (Fig. 5). In this case, first the sequence Phe-Leu-Arg-Asn- would originate from processing of the Antho-RNamide precursor, followed by an amino-terminal modification. The first step of this modification would be the conversion of the amino-terminal L-phenylalanyl into a 3-phenylpyruvyl group. This reaction could be catalyzed by an enzyme similar to phenylalanine dehydrogenase, which occurs in certain bacteria (27–29) and specifically catalyzes the deamination of L-phenylalanine:

$$\text{L-Phe} + \text{NAD}^+ + \text{H}_2\text{O} \rightleftharpoons \text{3-phenylpyruvate} + \text{NH}_3 + \text{NADH} + \text{H}^+.$$

Alternatively, the first step could be catalyzed by a transaminase (Fig. 5). The second step would be the conversion of an amino-terminal 3-phenylpyruvyl into an L-3-phenyllactyl group. This reaction could be catalyzed by an aromatic α-keto acid reductase-type of enzyme. Zannoni and Weber (30) have isolated an aromatic α-keto acid reductase from dog and beef heart that specifically catalyzes the reduction of 3-phenylpyruvate to L-3-phenyllactate:

$$\text{3-phenylpyruvate} + \text{NADH} + \text{H}^+ \rightleftharpoons \text{L-3-phenyllactate} + \text{NAD}^+.$$

This enzyme is different from lactate dehydrogenase and occurs not only in the heart but also in many other tissues, including the brain (30, 31). It should be stressed that all enzymes that might be involved in the conversion of the amino-terminal L-phenylalanyl into the L-3-phenyllactyl group should be able to accept a peptide-bound substrate instead of free L-phenylalanine and 3-phenylpyruvate. In addition, these enzymes should be localized in neurosecretory vesicles. If the first conversion step were catalyzed by an enzyme similar to phenylalanine dehydrogenase, there would be no need for an extensive NADH transport across the vesicle membrane, as the first oxidation step would yield the NADH necessary to carry out the second reduction. This would give the overall reaction

$$\text{L-phenylalanyl} + \text{H}_2\text{O} \rightleftharpoons \text{L-3-phenyllactyl} + \text{NH}_3.$$

In animals, phenylalanine is normally degraded by phenylalanine hydroxylase to form tyrosine, and not by an oxidative deamination or transamination as given in Fig. 5. In patients with phenylketonuria, however, where the hydroxylation step is blocked by absence or deficiency of phenylalanine hydroxylase, alternative pathways become evident: in addition to increased phenylalanine, also phenylpyruvate and phenyllactate are found in serum and urine (32). This suggests that the phenylpyruvyl group is a likely intermediate in the formation of the phenyllactyl group of Antho-RNamide.

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