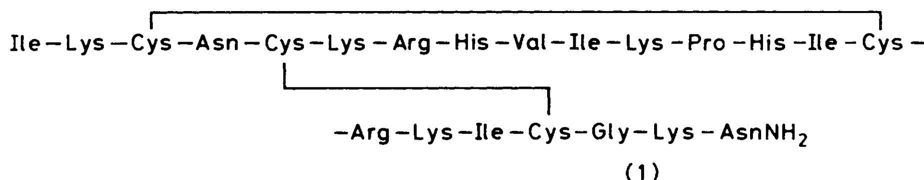
Selective Formylation of α- or ε-Amino-groups of Peptides

By **Christopher E. Dempsey,*** The Christopher Ingold Laboratories, University College, London WC1H 0AJ

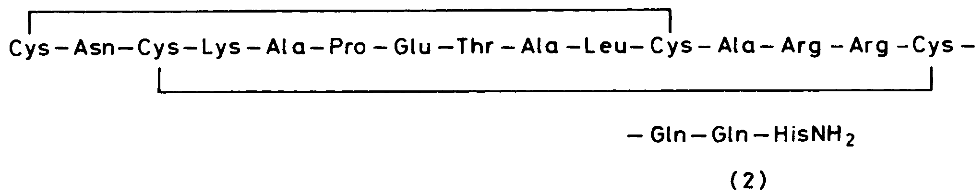
Derivatives of the bee venom peptides apamin and peptide-401 (MCD peptide) formylated selectively on the *N*-terminal (α-) amino-group, or on the ε-amino-group(s) may be isolated in high yields following formylation by two independent procedures.

PEPTIDE-401 (MCD peptide) is a highly basic peptide of 22-amino-acid residues (1) which is present in small amounts in the venom of the European honey bee (*Apis mellifera*).[1,2] The peptide is the most potent of a group of basic peptides [3] and synthetic polymers [4,5] which cause the degranulation of and release of histamine from rat peritoneal mast cells, an effect resulting in inflammatory responses on injection of peptide-401 into rats.[1] Paradoxically, peptide-401 is also a powerful anti-inflammatory agent, suppressing both adjuvant arthritis and carrageenan-induced hind paw oedema in the rat.[6]



(1)

The preparation of analogues of peptide-401 for studying the relationship between the structure of the peptide and its biological activities, and to introduce radioactive atoms into the structure of the peptide, is limited by the unusual amino-acid composition of (1). Peptide-401 has no free acid groups and no tyrosine residues (or other residues with aromatic side groups). Because methods for the modification of arginine and histidine residues suitable for the preparation of analogues for biological studies are limited by poor selectivity and the low stability of many of the resulting derivatives, chemically stable and well defined derivatives of peptide-401 are best prepared by modification of the amino-groups. In order to achieve some versatility in the modification of these groups, means of limiting reaction to selected residues is desirable. This is most likely to be achieved by selection between the *N*-terminal (α) amino-group and the lysine (ε) amino-groups by virtue of the different base strengths of these amino-groups in peptides. This report describes methods with which derivatives of peptide-401, formylated selectively on either the *N*-terminal amino-group or, alternatively, on the lysine ε-amino-groups, may be isolated. These procedures are shown to be applicable to the selective formylation of apamin (2), a second bee venom peptide of intense pharmacological interest.[7-11]



(2)

RESULTS AND DISCUSSION

Classically, amino-groups are formylated by the addition of acetic anhydride to a solution of the amine in formic acid.[12] N^{α}-Formyl-lysine is recovered when lysine is formylated in this way.[13] The method has been used for the preparation of N^{α}-formyl chemotactic peptides,[14,15] but the question of specificity in formylation between α- and ε-amino-groups did not arise because these peptides do not contain lysine.

Extensive formylation of peptide-401 is difficult to achieve using this method, even when high temperatures and high concentrations of acetic anhydride are used. However, a monoformyl derivative of peptide-401 was reproducibly prepared in good yield by treating the peptide in formic acid with low concentrations of acetic anhydride (a final proportion of 1 : 7 v/v acetic anhydride-formic acid). The monoformyl derivative was readily separated from small amounts of bisformyl, or unchanged peptide, by cation-exchange chromatography and was obtained in 60% yield. The product was homogeneous on high-voltage thin-layer electrophoresis at pH 6.5, and on thin-layer chromatography (t.l.c.), with mobility on t.l.c. of 1.8 relative to peptide-401 (the mobility of the bisformyl peptide is 2.3 relative to the native peptide). The degree of formylation of these peptides was confirmed by formylating in [^{14}C]formic acid. The calculated incorporation of [^{14}C]formyl groups was 0.85

* *Present address*: The Department of Biochemistry, Oxford University, South Parks Road, Oxford OX1 3QU.

and 1.68 for the mono- and bis-formyl peptides, respectively.

The position of the formyl group in the monoformyl derivative of peptide-401 was determined by analysing the tryptic fragments of the *S*-carboxamidomethylated peptide. Previous analytical mapping of trypsin digests of peptide-401 showed [16] that the *N*-terminal dipeptide, Ile-Lys, is the most mobile fragment on chromatography in butanol–pyridine–acetic acid–water (15 : 10 : 3 : 12, v/v). This fragment was absent from the digest of the monoformyl derivative and was replaced by a faster migrating peptide which was purified by preparative paper chromatography and shown to be formylated by detection of formylhydrazine following hydrazinolysis.[17] The amino-acid composition of the formyl fragment was Lys: 1.05, Ile: 1.00, and the position of the formyl group on the α-amino-group (rather than the ε-amino-group of Lys-2) was confirmed by *N*-terminal analysis. No formylhydrazine was detectable in the hydrazinolysates of the other trypsin fragments of the monoformyl peptide isolated by paper chromatography.

Previously,[18] incomplete cleavage by trypsin of the Lys-2-carboxymethylcysteine-3 peptide bond of *S*-carboxymethylated peptide-401 has been observed. Complete cleavage of this bond by trypsin occurred in the present study presumably because the cysteine thiol groups were blocked by carboxamidomethylation rather than by carboxymethylation.

A derivative of apamin (2), mono-formylated selectively on the α-amino-group by the addition of acetic anhydride to a solution of the peptide in formic acid, was isolated by cation-exchange chromatography in 70% yield and was homogeneous on t.l.c. (with mobility relative to apamin of 1.25). N^{ϵ}-Dansyl-lysine, but not dansyl-carboxymethylcysteine,* was found on *N*-terminal analysis of the *S*-carboxamidomethylated peptide. A sample of the *S*-carboxamidomethyl, monoformyl-apamin was reductively methylated.[19] Amino-acid analysis showed that the lysine was converted quantitatively into N^{ϵ}-methyl-lysine and N^{ϵ}-dimethyl-lysine, demonstrating that the ε-amino-group of the monoformyl peptide was free.

The selectivity for the α-amino-groups of peptide-401 and apamin on formylation in formic acid is due, at least in part, to the low concentration of the nucleophilic (unprotonated) form of the ε-amino-group(s) in formic acid. The formylation of ε-amino-groups in aqueous solution requires that the reaction be carried out at a higher pH where deprotonation of the ε-amino-group is favoured. The preparation of N^{ϵ}-formyl-lysine, by reaction of lysine with *p*-nitrophenyl formate at neutral pH, has been described.[20] Although it was later shown, by LeClerc and Benoiton,[21] that this selectivity was partly due to the high solubility of N^{α}-formyl-lysine which remained in solution on precipitation of pure N^{ϵ}-formyl-lysine, the latter authors showed that *p*-nitrophenyl formate was specific for the ε-amino-group of lysine at pH 11. In the present study the selectivity of *p*-nitrophenyl formate for the ε-amino-groups of peptides was examined at pH 9.5 to avoid the possibility of side reactions at the higher pH.

* Dansyl = 5-dimethylaminonaphthalene-1-sulphonyl.

Reaction of peptide-401 with *p*-nitrophenyl formate at a 5-fold molar excess over peptide amino-groups in cold borate buffer, pH 9.5, resulted predominantly in the pentaformyl derivative with a small amount of hexaformyl peptide. The degree of formylation of the peptides was determined by comparing their migration on t.l.c. with mixtures of *N*-formylated derivatives of peptide-401 containing one to six *N*-formyl groups. The characterization of the hexaformyl derivative was unambiguous because it was not detectable with the standard ninhydrin or fluorescamine reagents. The pentaformyl derivative was shown to be formylated only on the lysine (ε) amino-groups by the detection of dansyl-isoleucine but not N^{ϵ}-dansyl-lysine on *N*-terminal analysis at a peptide loading (10 nmol) at which less than 1% of free lysine is readily detectable.

Apamin was formylated at pH 9.5 with *p*-nitrophenyl formate at five-fold molar excess over peptide amino-groups in an ice-bath. The monoformyl derivative was isolated in 75% yield by cation-exchange chromatography and was homogeneous on t.l.c. with mobility of 1.48 relative to apamin. The position of the formyl group was determined as described for the characterization of N^{α}-formyl-apamin. Dansylcarboxymethylcysteine, but not N^{ϵ}-dansyl-lysine, was detected on *N*-terminal analysis of the *S*-carboxamidomethylated peptide, and no conversion of lysine into N^{ϵ}-methylated derivatives was found by amino-acid analysis following reductive methylation. The monoformyl derivative of apamin prepared by reaction with *p*-nitrophenyl formate at pH 9.5 is therefore formylated specifically on the ε-amino-group.

The selectivity of *p*-nitrophenyl formate for the ε-amino-group at high pH is presumably a consequence of the high nucleophilicity of the group which allows ammonolysis to complete with the rapid hydrolysis of *p*-nitrophenyl formate in alkaline solution (the half-life for the hydrolysis of *p*-nitrophenyl formate at pH 9.5 is of the order of a few seconds at 15 °C). Although the rate of hydrolysis of *p*-nitrophenyl formate is considerably slower at acid pH (half-life at pH 5 is *ca.* 9 min at 15 °C) selectivity for the α-amino-group could not be achieved with either peptide at low pH. In addition, reaction with the formylating reagent at pH 5—7 was complicated by the ability of the peptides to catalyse the hydrolysis of *p*-nitrophenyl formate, apparently as a result of base catalysis involving the imidazole groups of the peptide histidine residues (similar catalysis of *p*-nitrophenyl formate hydrolysis was observed with corresponding concentrations of imidazole).

Neither of the two formylating procedures were suitable for the preparation of derivatives of peptide-401 or apamin formylated at every amino group. Fully *N*-formylated derivatives of the peptides were readily prepared by the sequential use of both of the methods.

No detectable side reactions occur on formylation of peptide-401 or apamin using either procedure. Both

peptides are stable to extended periods in formic acid at room temperature (retaining full biological activity) and no reduction of disulphide bonds was detectable (by using Ellmans reagent [22]) in alkaline solutions up to pH 10.5. The inability to detect formylhydrazine in hydrazinolysates of the trypsin fragments other than the *N*-terminal dipeptide after formylation of peptide-401 in formic acid, demonstrates that the imidazole nucleus was not formylated. Similarly, no *o*-formylation of the threonine hydroxy-group of apamin was detected under either of the formylating conditions; *o*-formyl groups are rapidly saponified at alkaline pH.[23]

The selective formylation of the *N*-terminal amino-group of peptide-401 has little effect either on the anti-inflammatory activity or on the mast-cell-degranulating activity of the peptide. Both activities are abolished on formylation of each of the six amino-groups of peptide-401. All *N*-formyl derivatives of apamin retain full biological activity (both neurotoxic [1] activity and also action on certain smooth muscle preparations [10]), although activity in some of the *N*-formyl derivatives of apamin may be a result of deformylation during biological assay.[24]

The specificities of the two formylating procedures are not absolute, selectivity for the α- or ε-amino-group resulting from the increased kinetic reactivity of one type of amine over the other under particular reaction conditions. The desired derivative must, therefore, be separated from small amounts of unchanged or 'over-reacted' peptide. However, the suppression of charge on formylation of amino-groups, together with the low levels of side reactions allows the purification of selectively formylated derivatives of the peptides in high yields by ion-exchange chromatography. This selectivity may be useful in determining separately the effect of modification of α- or ε-amino-groups in other biologically active peptides. Formylation is intrinsically the most satisfactory means of suppressing the charge on polypeptide amino-groups because it results in minimal increase in steric bulk at the amino-group. The mild conditions under which ε-amino-groups are formylated with *p*-nitrophenyl formate may make the reagent suitable for the formylation of proteins, which are unlikely to be stable to reaction in formic acid.

The ability to formylate α- or ε-amino-groups selectively leads to the possibility of introducing selectivity into otherwise non-selective modification procedures (*e.g.* reductive methylation [25]) that are stable to the conditions required to deformylate peptide amino-groups (0.1M methanolic hydrochloric acid at room temperature for 24 h [23]). Apamin is a rather severe test of the applicability of the formyl group to the reversible selective protection of amino-groups in native peptides, having four terminal amide groups susceptible to deamidation under these deformylation conditions. In practice the rate of deformylation was sufficiently greater than the rate of de-amidation of the side group and *C*-terminal amide groups of apamin to allow the isolation (in low yields) of derivatives of the peptide methylated selectively on the α- or the ε-amino-groups by the procedure of reductive methylation of selectively formylated derivatives followed by deformylation.

EXPERIMENTAL

Analytical Methods.—Thin-layer chromatography was carried out using Merck cellulose plates. The chromatograms were developed for about 18 h in butanol–pyridine–acetic acid–water (15 : 10 : 3 : 12, v/v). Analytical electrophoresis was carried out using cellulose thin-layer plates in pyridine–acetic acid buffer, pH 6.5 (60 V cm^{-1} for 30 min). Samples for amino-acid analysis were hydrolysed in 6M hydrochloric acid at 105 °C under vacuum for 24 h, and then analysed using a Technicon AutoAnalyzer. The extent of formylation of ε-amino-groups was quantitated in some cases by reductive methylation to produce acid-stable N^{ϵ}-methylated derivatives of unformylated lysine residues. The peptide (0.4 mg, 2 × 10^{-8} mol) was dissolved, at 4 °C, in phosphate buffer (0.05M, pH 6.8, 0.2 ml; containing 5mM nickel acetate). Sodium cyanoborohydride (0.4 mg, 6.3 μmol) in 25 μl of phosphate buffer, and 10% aqueous formaldehyde (2 μl, 6.7 μmol) were added, and the solution was stirred for 24 h at 4 °C. The peptide was recovered by gel filtration (Sephadex G-10, 0.9 × 10 cm) and lyophilization, and analysed for N^{ϵ}-methyl-, and N^{ϵ}-dimethyl-lysine by amino-acid analysis after calibration of the column with standard N^{ϵ}-methyl derivatives of lysine.

N-Terminal analysis was carried out by the method of Hartley,[26] loading an amount equivalent to 10 nmol of dansylated-peptide hydrolysate onto the polyamide thin-layer plate to ensure that levels of free (unformylated) amino-groups above 1% were detected (0.1 nmol of dansyl-amino-acid is readily detectable). The *N*-terminal cysteine residue of apamin was protected as carboxamidomethyl-cysteine before analysis. The peptide (0.2 mg, 10^{-8} mol) was dissolved in 0.2 ml of degassed Tris-HCl buffer (0.5M, pH 8.5) containing 6M urea and dithiothreitol (0.3 mg, 1.9 μmol), and incubated under nitrogen at room temperature for 4 h. Recrystallized iodoacetamide (0.75 mg, 4 μmol) in 40 μl of Tris-HCl buffer, was added and the solution left for 20 min in the dark. The peptide was recovered by gel filtration (Sephadex G-15, 0.9 × 15 cm) in 2% acetic acid in the dark and lyophilization, before *N*-terminal analysis.

Peptides were analysed for the presence of formyl groups by using the qualitative assay of Narita.[17] After hydrazinolysis of the formyl peptide or trypsin fragment in anhydrous hydrazine (98 °C for 1 h), and evaporation of hydrazine under reduced pressure (over P_2O_5 and H_2SO_4), hydrazinolysates were dissolved in 50% aqueous ethanol, and an amount equivalent to 0.5 mol of peptide was spotted onto Whatman 3MM paper. The paper was developed by descending chromatography in pyridine–aniline–water (9 : 1 : 4, v/v) for *ca.* 8 h. Acyl-hydrazines were detected by spraying with alkaline silver nitrate (0.1M $AgNO_3$–5M ammonia solution, 1 : 1, v/v) and identified by comparison with standard acyl-hydrazines, formylhydrazine, R_F 0.64 and acetylhydrazine R_F 0.71, (prepared as described by Mashima [27]). The detection limit of the assay was *ca.* 10^{-8} mol of formylhydrazine.

Isolation of the Peptides from Bee Venom.—Peptide-401 and apamin were isolated from lyophilized bee venom by forced dialysis, gel filtration on tandem columns of Sephadex G-25 and G-50, and ion-exchange chromatography on SP-Sephadex C-25 as described by Gauldie *et al.*,[2] followed by

chromatography on Heparin-Sepharose CL-6B as described by Banks *et al.*[26] The peptides were homogeneous on electrophoresis and t.l.c. in the systems described above. A single *N*-terminal amino-acid was found on *N*-terminal analysis (dansyl-isoleucine for peptide-401, and dansyl-carboxymethylcysteine for apamin), and the peptides had the correct amino-acid composition, within experimental error, on amino-acid analysis.

Formylation of the Peptides in Formic Acid.—Peptide-401 (30 mg) was dissolved in 98% formic acid (700 μl) at 20 °C and acetic anhydride (20 × 5 μl aliquots) was added over a period of 10 min to the stirred solution. After a further hour, distilled water (2 ml) was added and the peptide was twice lyophilized from distilled water. The peptide was fractionated by cation-exchange chromatography on SP-Sephadex C-25 (0.9 × 11 cm) in 0.1M sodium acetate buffer, pH 4.7, eluting with a salt gradient (0.4—0.8M NaCl, 100 ml at 5 ml h^{-1}). Mono-(N^{α})-formylpeptide-401 (18.5 mg, 62%, after desalting and lyophilization) was eluted at a salt concentration of *ca.* 0.65M sodium chloride, and bisformylpeptide-401 (2.5 mg, 8%) was eluted at a salt concentration of *ca.* 0.57M sodium chloride. Radiolabelling of the peptides was carried out in the same way, except that the formic acid contained 0.85 mCi of sodium [$^{14}C_1$]formate (Radiochemical Centre, Amersham; 54.6 mCi $mmol^{-1}$) giving 46 μCi $mmol^{-1}$ as the specific activity of the formic acid. The specific activities of the labelled peptides were 40.3 and 75.6 μCi $mmol^{-1}$ for the mono- and bis-formyl derivatives of peptide-401, respectively, as determined by scintillation counting. Apamin (20 mg) was formylated as described for the monoformylation of peptide-401, by the addition of 12 × 5 μl aliquots of acetic anhydride to a solution of the peptide in 98% formic acid (400 μl). The peptide was fractionated on SP-Sephadex C-25 (0.9 × 14 cm) as described above, except that a salt gradient of 0—0.5M sodium chloride was used to elute the peptides. N^{α}-Formylapamin (13.5 mg, 68% after desalting) was eluted at a salt concentration of about 0.3M sodium chloride, and N^{α},N^{ϵ}-(bis)formylapamin (2.2 mg, 11%) was eluted at about 0.2M sodium chloride.

Formylation of the Peptides with p-*Nitrophenyl Formate.*—*p*-Nitrophenyl formate was prepared by the dicyclohexylcarbodi-imide mediated condensation of *p*-nitrophenol with formic acid, in tetrahydrofuran.[20] The twice recrystallised product had a melting point of 71—73 °C (lit.,[20] 72—74 °C). Apamin (10 mg, 5 μmol) was dissolved in 1.5 ml of sodium tetraborate buffer (0.1M, pH 9.5) in an ice-bath. *p*-Nitrophenyl formate (4 mg, 24 μmol) in tetrahydrofuran (60 μl) was added to the stirred solution. After 10 min, the solution was acidified with formic acid until the yellow colour (*p*-nitrophenoxide anion) had disappeared, and then extracted with diethyl ether (3 × 1.5 ml) to remove *p*-nitrophenol (alternatively, *p*-nitrophenol may be removed by gel filtration). The solution was diluted, and then applied directly onto a column of SP-Sephadex C-25 (0.9 × 14 cm). The peptides were eluted as described for N^{α}-formylapamin, using a salt gradient (0.0—0.4M NaCl). N^{ϵ}-Formylapamin (7.3 mg, 73% after desalting and lyophilization), and N^{α},N^{ϵ}-diformylapamin (1.0 mg, 10%) were eluted at salt concentrations of *ca.* 0.3 and 0.2M sodium chloride, respectively. Peptide-401 (10 mg, 3.8 mol) was formylated with *p*-nitrophenyl formate (20 mg, 0.12 mmol) in 2 ml of borate buffer, as described for apamin. Following extraction of *p*-nitrophenol from the acidified solution, the peptide was recovered by gel filtration (Sephadex G-15), and lyophilized T.l.c. showed that the product was mainly the pentaformyl derivative with a small amount of (ninhydrin-unreactive) hexa-*N*-formyl-peptide-401, by comparison of the migration of the peptides with mixtures of derivatives of peptide-401 formylated at one to six of the amino-groups (prepared in a series of reactions at varied pH and *p*-nitrophenyl formate concentration).

Trypsin Digestion of N^{α}-*Formyl-peptide*-401.—Peptide-401 and the monoformyl derivative prepared by reaction in formic acid (3 mg of each), were separately reduced and carboxamidomethylated with iodoacetamide as described above. The *S*-carboxamidomethyl peptides (1.8 mg) were dissolved in distilled water (1 ml) and the solution was adjusted to *ca.* pH 8 with 0.01M NaOH. Trypsin (Sigma, TPCK-treated; 80 μl of a 1 mg ml^{-1} solution adjusted to pH 8) was added and the solutions incubated at 37 °C for 3 h. The proteolysis products were recovered by lyophilization, and analysed for the presence of formyl groups as described above, after purification by preparative paper chromatography (Whatman 3MM in butan-1-ol–pyridine–acetic acid–water; 15 : 10 : 3 : 12, v/v).

Deformylation of Selectively Formylated Derivatives of Apamin Following Reductive Methylation.—N^{α}-Formylapamin (7 mg, 3.5 mol) was methylated on the ε-amino-group by reductive methylation as described under *Analytical Methods* (repeating the reaction if necessary to ensure essentially complete dimethylation of the amino-group). Following gel filtration and lyophilization, the peptide was dissolved in methanolic hydrochloric acid (0.1M; 2.4 ml) and left for 18 h at room temperature. The peptide was recovered by repeated lyophilization from distilled water and then dissolved in sodium tetraborate buffer (0.1M; pH 9.8) and left for 24 h at room temperature (this treatment regenerates the free acid group of the Glu-7 residue which is esterified in methanolic HCl). Following gel filtration the selectively methylated derivative was isolated by ion-exchange chromatography (SP-Sephadex C 25, 0.9 × 13 cm; eluting with a salt gradient of 0—0.6M sodium chloride in sodium acetate buffer, pH 4.7; 100 ml). The yield of N^{ϵ}-dimethylapamin was 1.4 mg (20% from N^{α}-formylapamin); N^{α}-dimethylapamin (0.8 mg; 13% from 6 mg of N^{ϵ}-formylapamin) was prepared in a similar manner. The peptides were homogeneous on t.l.c. and were ninhydrin-reactive; the positions of methylation were confirmed by *N*-terminal analysis and amino-acid analysis. N^{α}-Dimethylapamin could be formylated with *p*-nitrophenyl formate to produce a peptide indistinguishable from the product obtained on reductive methylation of N^{ϵ}-formylapamin; similarly, formylation of N^{ϵ}-dimethylapamin in formic acid resulted in a derivative indistinguishable from that obtained on reductive methylation of N^{α}-formylapamin. The low yields were mainly a result of de-amidation of side chain and *C*-terminal amide groups as determined by the ability of the less basic components eluting from the ion-exchange column to incorporate greater than one methyl group on esterification in methanolic hydrochloric acid. Other possible side reactions, the reduction of disulphide bonds or an N $\longrightarrow$ O acyl shift involving the Thr-9 residue, were shown to be negligible by using Ellman's reagent [22] and by *N*-terminal analysis, respectively.

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