FORMATION OF OPEN-CHAIN ASYMMETRICAL CYSTINE PEPTIDES ON A SOLID SUPPORT. SYNTHESIS OF pGLU-ASN-CYT-PRO-ARG-GLY-OH

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Dedicated to the memory of Dr Karel Bláha.

The feasibility of the synthesis of asymmetric disulfide-containing peptides on solid phase resins was investigated. Using a fragment of [8-arginine]vasopressin as a model, the conversion of the S-acetamidomethylcysteine-containing peptide-resin into the corresponding S-carboxymethylsulfenyl derivative followed by reaction with free cysteine was studied. Both reactions proceeded smoothly under mild conditions. After cleavage from the resin the peptide was shown to have the correct composition.

The neurohypophyseal nonapeptide hormones [8-arginine]vasopressin (AVP)* and oxytocin (OXT) are long known for their peripheral, hormonal activities. Inspired by the successful first synthesis of these nonapeptides by du Vigneaud in 1954 (ref.[1]), numerous analogues have been synthesized and tested for oxytocic, pressor and antidiuretic activity[2,3]. Many agonists and antagonists of these responses with improved selectivity of action are known[2,3]. In addition, AVP and OXT were found to have effects on behavioural processes; fragments of these peptides retain central activities whereas they are practically devoid of endocrine activity[4,5]. Incubation of AVP and OXT with a rat brain synaptic membrane preparation results in fragments with an intact disulfide bridge[6]. These peptides, of which the 4-9 and 4-8 fragments have a pyroglutamic acid residue as the N-terminal end[6], were found to be more potent than the parent molecules[7–9].

We have described the synthesis of several of these fragments containing a cystine residue in position 6 (an asymmetrical disulfide) by treatment of the

* Standard abbreviations are used for amino acids and protecting groups [IUPAC–IUB Joint Commission on Biochemical Nomenclature, J. Biol. Chem. *260*, 14 (1985)]. Other abbreviations are: Acm, acetamidomethyl; Cyt, cystine; MSA, methanesulfonic acid; Npys, 3-nitro-2-pyridinesulfenyl; Pms, pentamethylbenzenesulfonyl; Scm, methoxycarbonylsulfenyl.

pertinent, protected Cys(Trt)-containing peptides with methoxycarbonylsulfenyl chloride followed by reaction with the free thiol function of cysteine[10,11]. It proved also possible to obtain the 4-9 and 4-8 fragments with cystine in position 6 in good yield and purity starting from the corresponding free (symmetrical) dimers by treatment with Scm-Cl followed by reaction with cysteine[12]. In this communication we wish to report our results of synthesizing this type of asymmetrical disulfide containing peptides on a solid phase resin.

It is known that amide bond formation via side-chain functions on the resin to give cyclic peptides is feasible, be it that depending on the desired ring size a mixture of cyclic structures is formed[13,14]. Cyclization via disulfide bridge formation in neurohypophyseal hormones was described already in 1968 (ref.[15]), but this approach was later used very rarely (for the review of these attempts see ref. [16]). Only very recently an elegant method presented by Mott et al.[17] was brought to our attention. In this publication a Cys(Acm) residue is introduced in the growing peptide chain (benzhydrylamine resin) and converted into the corresponding Scm-peptide one step prior to the introduction of the second cysteine residue which was incorporated as (the N-terminal) Z-Cys(Trt). Acid treatment with TFA (75% with 15% CH_2Cl_2 and 10% anisole) apparently only removes the Trt function and is followed by a 16 h intramolecular cyclization in N-methylmorpholine–CH_2Cl_2 (1:19, v/v)[17]. No yields on the separate steps have been reported. Recently two more papers have appeared on cyclization via disulfide bridge formation between two cysteine residues, while the otherwise (partially) protected peptides were still linked onto the resin[18,19]. In the first case a tert.butyl group was removed from Cys leaving an Npys function intact for S—S bridge formation[18], while in the other report two S-tert.butyl groups were removed and potassium ferricyanide in DMF/water was added in order to form the ring structure[19].

Here we want to describe a similar approach for intermolecular disulfide bond formation as in our solution syntheses performing, however, the reactions on the resin. The only difference is that we used the Acm group instead of the Trt group for SH protection; the formation of the methoxythiocarbonate derivative is unaffected. Similar results have been found with other molecules in solution synthesis (ref.[20] and unpublished observations). pGlu-Asn-Cyt-Pro--Arg-Gly-OH, i.e. [pGlu^4, Cyt^6]AVP-(4-9) acid, was used as a model compound.

The synthesis of the peptide derivative pGlu-Asn-Cys(Acm)-Pro--Arg(Pms)-Gly, linked to a *p*-alkoxybenzyl alcohol resin proceeded as expected. The loading of the resin with the desired compound was estimated at 0.20 mmol/g by cleavage of some of the material from the support, followed by HPLC (main component 69%) and amino acid analysis. The amounts of

Scm-Cl and cysteine to be used in subsequent reactions were, however, related to the loading of the starting Fmoc-Gly-resin, 0.26 mmol/g, and were therefore somewhat higher than planned. TLC and HPLC analyses of crude samples, obtained by cleavage of some material from the support, indicated that reaction of the Cys(Acm)-containing peptide-resin with 4 equivalents of Scm-Cl for 30 minutes cleanly converted the Acm-protected thiol into an Scm-activated function. In the next reaction the resin-bound Cys(Scm) peptide is transformed into the cystine-compound, i.e. an open-chain asymmetrical disulfide, using 2 equivalents of cysteine; the reaction was complete within 30 minutes as determined by TLC and HPLC. It is interesting and important to note that H-Cys-OH.HCl apparently has rapid and good access to the hydrophobic environment of the polystyrene resin, and that intermolecular disulfide bridge formation is much more rapid than the corresponding intramolecular reaction[17]. Prolonged reaction times (1 and 2 h) did not result in increased yield or decomposition of the desired product: in each case the product peak in the crude peptide amounts to 35–40% according to HPLC, while the amount of the major byproduct, possibly the symmetrical peptide disulfide, is 10–14%. In view of the amount of pGlu-Asn-Cys(Acm)-Pro-Arg(Pms)-Gly-OH present in the crude product before conversion into the Cyt-peptide (69% according to HPLC), it can be concluded that the desired resin-bound open-chain asymmetrical disulfide is formed in 50–60% yield. In spite of possible contaminants in and/or interference by the solid matrix, these data correlate well with those found for disulfide formation in solution by similar methods, where 2.5 equivalents of Scm-Cl and 1.5–2 equivalents of cysteine are used[11].

Purification of the cystine-containing peptide bearing a Pms-function was achieved by column chromatography. Acidolytic removal of the Pms-group was accompanied by the formation of some minor byproducts that were removed by column chromatography. The purity and identity of the product were established by TLC, HPLC, amino acid analysis and fast atom bombardment mass spectrometry. Simultaneous cleavage of this peptide from the resin and the Pms group is of course possible. However, we chose a two-step approach since with other peptides separate deprotection has sometimes resulted in easier purification than that after simultaneous cleavage. In addition, the Pms-containing peptide was needed for reference purposes.

It can be concluded that the method described here for the preparation of open-chain asymmetrical cystine peptides on a solid support can be a valuable addition to procedures described in the literature for the solid-phase synthesis of cyclic disulfides[15–19]. The synthesis requires relatively mild reaction conditions and provides the asymmetrical disulfides in good yield.

EXPERIMENTAL

The purity of the amino acid derivatives and peptides was checked by thin layer chromatography on Merck silica gel plates (F 254 nm, 0.25 mm) in the solvent systems 1-BuOH–AcOH–H_2O (4 : 1 : 1, S1), 1-BuOH–pyridine–AcOH–H_2O (8 : 3 : 1 : 4, S2), and 1-BuOH–AcOH–H_2O (2 : 1 : 1, S3). UV, ninhydrin and chlorine/*o*-tolidine were used for the detection of compounds on TLC-plates. Amino acid analyses were carried out on samples that had been hydrolyzed in 5.7M HCl for 24 h at 105°C (Kontron Liquimat III apparatus). The presence of residual TFA and MSA as well as the quantitative determination of acetic acid was established in a single run by isotachophoresis[21]. HPLC assays were carried out on a Spectra Physics apparatus, model 8000. Stationary phase: Supelcosil LC-18-DB. As mobile phase gradients of acetonitrile in 0.1M NaH_2PO_4, pH 2.1 were used. Detection was at 210 nm, flow rate 1 ml min and temperature 35°C. The percentage of the main component is given.

Solid phase synthesis: The peptide was synthesized on a *p*-alkoxybenzyl alcohol (Wang) resin using 3 equivalents of Fmoc-amino acid, 4.5 equivalents of HOBt and 3.3 equivalents of DCC. Side-chain protective groups were Pms for arginine[12] and Acm for cysteine. The N-terminal pyroglutamic acid residue was introduced without protection of the pyrrolidone NH. Completeness of coupling reactions was monitored by qualitative Kaiser tests and double acylations were carried out where necessary; a standard double acylation was used at the proline residue. Capping steps with acetic anhydride–pyridine (1:2, v v) were carried out before removal of the N^{α}-Fmoc group, thus eliminating possible formation of deletion sequences. Using these procedures we obtained pGlu-Asn-Cys(Acm)-Pro-Arg(Pms)-Gly-resin. Amino acid analysis of a sample obtained by cleavage from the support indicates a loading of the desired peptide of 0.20 mmol g resin, while the loading of the starting Fmoc-Gly-resin was 0.26 mmol g.

On-support transformation of Cys(Acm)- *into* Cyt-*peptides*: The Cys(Acm)-residue in the peptide-resin was converted into the Cys(Scm)-derivative by reaction with 4 equivalents of Scm-Cl in MeOH–CH_2Cl_2 (1:1, v v) for 30 min. Subsequent reaction of the Cys(Scm)-containing peptide resin with H-Cys-OH.H_2O.HCl (2 equivalents) in DMF for various periods of time (0.5, 1.0, and 2.0 h) provided the desired pGlu-Asn-Cyt-Pro-Arg(Pms)-Gly-resin. Both reactions were carried out at room temperature.

Cleavage of peptides from the resin: Peptide derivatives were cleaved from the support by treatment of the peptide-resin with TFA–CH_2Cl_2 (1:1, v v) for 2 h at room temperature. The crude products were isolated in 80–90% yield by evaporation to dryness followed by dissolution of the residue in DMF and precipitation in ether. Samples were analyzed by HPLC.

pGlu-Asn-Cyt-Pro-Arg(Pms)-Gly-OH

The title compound was purified on silica gel (Merck Fertigsäule) using the solvent system 1-BuOH–AcOH–H_2O (3:1:1, v v). The product was isolated by evaporation of the appropriate fractions to dryness followed by lyophilization of the residue from t-BuOH–H_2O (1:1, v v). TLC: R_F 0.40 (S1), 0.40 (S2); HPLC: 88.9%.

pGlu-Asn-Cyt-Pro-Arg-Gly-OH

Removal of the Pms group was performed by treatment of the peptide derivative with a mixture of TFA–MSA–thioanisole (10:1:1, v v), concentration 3 wt.%, for 16 h at 4°C. The crude product was obtained by dropwise addition of the reaction mixture to ether, centrifugation, washing with ether and drying. Purification was achieved by chromatography on silica gel (Merck Fertigsäule) using

the solvent system 1-BuOH–AcOH–H_2O (2:1:1, v/v). Pooling of product-containing fractions followed by evaporation to dryness and lyophilization from water, after exchange for acetate ions, provided the title compound in 79% yield. TLC: R_f 0.15 (S1), 0.24 (S2), 0.13 (S3); HPLC: 96.0%. Amino acid analysis: Asp 1.05, Glu 1.02, Pro 1.06, Gly 1.00, Cyt 1.00, Arg 0.99, NH_3 1.07. Isotachophoresis: AcOH 2.1%, residual TFA 0.5%. The molecular mass (755) was confirmed by fast atom bombardment mass spectrometry.

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