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Substituted Benzhydrylamine Resins in Solid Phase Peptide Synthesis of Peptide Amides and Peptides with C-terminal Asparagine monitored by Potentiometric Titration with Perchloric Acid. Classification of Acid Lability of Different Resins

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The acid lability of the bond between a benzhydrylamine resin and an amino acid residue was determined by potentiometric titration with perchloric acid in acetic acid for methyl- and methoxy-substituted benzhydrylamine resins with 1 % and 2 % cross-linking, respectively, in experiments with alanyl and *β*-aspartyl resins by successive treatments with 1 M HCl in acetic acid or 5 M HBr in acetic acid. In the synthesis of the resins only the Leuckart reduction method of the primary ketoresin yielded a usable product. It has been shown that the usual solid phase procedure including the perchloric acid monitoring method could be used if the extent of substitution is comparable with those of the normal Merrifield resins. In a synthesis of a peptide with a *C*-terminal asparagine moiety the most convenient synthetic route was *via* the benzhydrylamine resin.

Solid phase synthesis of peptides is a well-established method for the preparation of many peptides and fragments of longer peptides, both protected and deprotected.[1] Most reports deal with peptides with a free *C*-terminal carboxyl function synthesized with the *C*-terminal residue anchored to the resin by an ester bond of the benzyl type. The cleavage conditions are strong acids such as liquid HF or hydrogen bromide and trifluoroacetic acid.[2]

The possibility to cleave the peptides as fully protected peptides for synthesis of peptide amides or as fragments with a free *C*-terminal carboxyl group for synthesis of longer peptides exists.[3] The peptide amides especially are of interest as several biologically active peptides are of that type.[4-6] These methods use moderately strong bases as triethylamine – methanol, saturated ammonia in methanol or 2-dimethylaminoethanol.[3] Unfortunately the basic cleavage methods are slow in most cases and very sensitive to steric factors in the *C*-terminal region of the peptide chain. Therefore side-reactions can be favoured, *e.g.*, racemization or modification of side-chain protecting groups. Addition of catalysts to speed up the reaction, *e.g.*, potassium cyanide[7] or thallium(I) ethoxide[8] are of some importance, but the side-reactions mentioned still exist and may even become more pronounced.

A direct method of solid phase synthesis with cleavage of the peptide as an amide is therefore desirable. The most common procedure makes use of an acid-labile amide protecting group linked to the solid support, *e.g.*, a benzhydrylamino group.[9] The first resin of this type was synthesized with an unsubstituted benzhydrylic anchor. The peptide amide could therefore only be cleaved by very strong acid, such as liquid HF. If a weaker acidic environment is desired an *ortho* or *para* substitution at the aromatic ring (see Fig. 1) with an electron-donating group may be performed.[10]

A more special synthetic problem that can be

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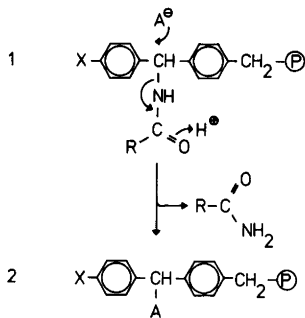


Fig. 1. Formulae 1–2 show the cleavage of the peptide–amino acid as the amide from the benzhydrylamine resin by an acid. R = $-CH(NH_2)CH_3$ (Ala), $-CH_2CH(NH_2)COOH$ (β-Asp); X = $-OCH_3$, $-CH_3$; A = Br, F.

solved by use of this type of support is synthesis of peptides with *C*-terminal asparagine or glutamine. These two amino acid residues have been very difficult to esterify to normal chloromethylated resins of the Merrifield type without side-reactions. Esterification with bases, *e.g.*, triethylamine in ethanol results in partial alcoholysis of the side-chain amide. Protection of the side-chain amide function with bulky groups, *e.g.*, 2,4,6-trimethoxybenzyl- (Tmb),[11] bis-(2,4-dimethoxybenzyl)-(Dmb_2)[11] or 4,4′-dimethoxybenzhydryl- (Mbh)[12] results in low yield in the esterification to the resin[13] and consequently a considerable residue of unreacted, potentially active chloromethyl groups.[14–15] These two residues can be bound to the benzhydrylamine resin through their side-chains and cleaved directly by acid methods to the amides.[16–17] Finally there is the possibility to perform bidirectional synthesis if asparagine or glutamine are not *C*-terminal residues.

In this investigation the utility of the substituted benzhydrylamine resins in a usual solid phase procedure was examined. Two parameters in the evaluation are the acid lability to the peptide cleavage reagent and the acid stability to the deblocking reagent for the α-amino protecting group. These two parameters were calculated from stability and cleavage experiments with alanyl and β-aspartyl resins, substituted at the aromatic part of the benzhydryl group with either a methoxy or a methyl group both on 1 and 2 % cross-linked polystyrene–divinylbenzene material, respectively.

In the synthesis of the benzhydrylamine resins it was necessary to reinvestigate the synthetic routes so far published.[9,10,18]

In solid phase peptide synthesis it is essential to check every step to unveil incomplete coupling or deblocking steps. For automated synthesis the best monitoring procedure seems to be the perchloric acid titration method.[19] It was also of importance to examine to what extent this monitoring method could be used in peptide synthesis on the benzhydrylamine resins.

Two examples of peptide synthesis on selected resins were performed and monitored by the potentiometric titrations with perchloric acid for the first time. The two fragments were chosen from biologically relevant peptides with residues demanding bulky protecting groups in the side-chains.

The first peptide with the sequence Val-Met-Thr-Glu-Ala-NH_2 is the *C*-terminal part (14–18) of the neurosecretory peptide hormone from the shrimp *Pandalus borealis*, the distal retinal pigment hormone (DRPH),[20] that regulates the light adaption by action on special pigment cells in the eyestalks of the animal.[21] The obvious problem with classical solid phase synthesis, where the peptide had to be cleaved from the resin by ammonolysis is the tendency to undergo side-reaction with the protected glutamic acid residue, as this residue will be converted to a glutamine residue through ammonolysis of the ester protecting group.

The second peptide chosen was the *C*-terminal tetrapeptide of the A-chain of insulin (18–21) Asn-Tyr-Cys-Asn, where the problematic asparagine should be linked to the resin. In the synthesis of this sequence on the benzhydrylamine support the first coupled amino acid derivative was *N*-Boc-α-benzyl aspartate.

In both cases, a synthesis of the same sequence on the usual Merrifield resin was performed to compare the progress of the syntheses on the two types of resins followed by potentiometric titration with perchloric acid.

EXPERIMENTAL

Methods

Potentiometric titration. The potentiometric titrations with 0.05 M perchloric acid in acetic acid were carried out on a Radiometer equipment as end-point titrations.[19] As solvent, a mixture of acetic acid–dichloromethane (1:1 v/v) with a solvent–resin ratio equal to 10 ml/g was used. In some ambiguous

cases with drifting potentials 3 ml of 0.1 M tetrabutylammonium perchlorate in dichloromethane was added.[22]

Solid phase peptide synthesis was carried out in a manually operated reaction vessel. The ratio solvent–resin was kept constant at 10 ml/g resin. All couplings on the resin were performed with *N*,*N'*-dicyclohexylcarbodiimide (DCC) as the coupling reagent in two successive steps, (1) using only 1.5 × eqw. amount of the theoretical amount for 2 h, (2) repeated coupling with 1 × eqw. overnight. In the synthesis with the ester resin-bound peptide the first Boc-amino acid was introduced as the Cs-salt in a reaction with a chloromethylated S-X1 or S-X2 polystyrene–divinylbenzene resin suspended in *N*,*N*-dimethylformamide at 50 °C.[23] In the synthesis with the amide resin-bound peptide the first coupling to the resin was carried out as a normal DCC-coupling.

The α-amino protecting group (Boc-) was cleaved by 1 M HCl in acetic acid. A complete cycle involved: (1) Titration with 0.05 M perchloric acid, (2) cleavage of the Boc-group, (3) titration and (4) coupling of the next Boc-amino acid. In some cases a repeated coupling or deblocking was necessary to complete the step (see Figs. 3 and 4).

Stability experiments with the benzhydrylamine resins. An aliquot of the resin (1.5–2.5 g) was subjected to the following cycle: (1) Titration (to determine the extent of substitution), (2) coupling with the Boc-amino acid derivative (Boc-Ala or Boc-Asp-α-OBzl), (3) titration (zero value), (4) deblocking of the Boc-group with 1 M HCl in acetic acid, (5) titration, (6) treatment of the resin again with deblocking reagent for selected times, (7) titration and (8) repetition of (6)–(7). The combined filtrates from cycles Nos. 4 and 6 were collected and subjected to amino acid analysis (see Fig. 2).

Cleavage experiments with the benzhydrylamine resins. In the cleavage experiments the cycles were identical with the above-mentioned with the exception of Nos. 4 and 6 where 1 M HCl in acetic acid was replaced by 5 M HBr in acetic acid (see Fig. 2).

Determination of secondary amine in the resins by the Demjanov method.[10] The resins were analyzed for the content of nitrogen before and after treatment with nitrous acid. The results are summarized in Table 1.

Amino acid analysis were carried out on a Beckman Multichrom Liquid Column Chromatograph 4255 after previous hydrolysis of the samples in sealed ampoules. The cleaved peptides were hydrolyzed for 24 h in 6 M HCl and the resin-bound peptides in a mixture of 6 M HCl–acetic acid (1:1 v/v) for 48 h.

Thin layer chromatography was performed on precoated plates (Silicagel 60 F-254) from E. Merck. The following solvent systems were used: S2: 2-butanol–formic acid–water 75:15:10; S4: 1-bu-

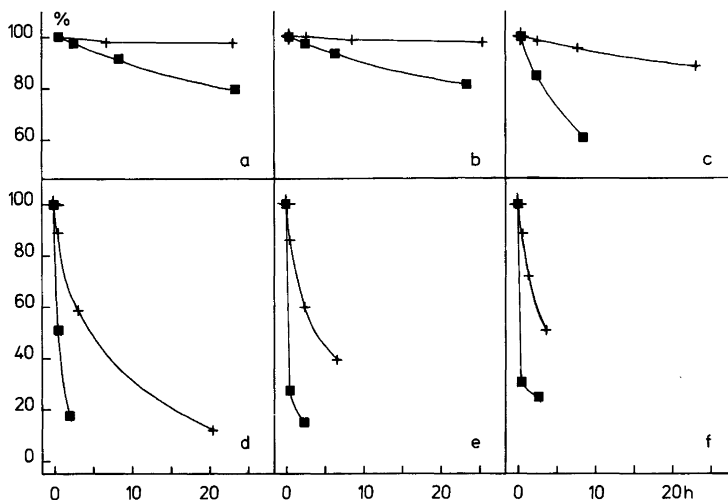


Fig. 2. Perchloric acid titration of the benzhydrylamine resins after treatment with 1 M HCl in acetic acid, Boc-group cleavage condition (2a–c) and 5 M HBr in acetic acid, peptide release condition (2d–f). + are values for the methyl-substituted benzhydrylamine resins and ■ values for the methoxy-substituted resins. 2a, d show the values for the alanyl-resins of the S-X1 type; 2b, e the same for resins of the S-X2 type and 2c, f for the β-aspartyl resins of the S-X2 type.

Table 1. Determination of secondary amine in the resins by the Demjanov method.

Resin	Reduction method	Nitrogen %	Total amine meqv g^{-1}	Titration[c] meqv g^{-1}	Nitrogen[d] %	Secondary amine meqv g^{-1}	Primary amine[e] meqv g^{-1}
3	*a*	1.870	1.335	0.708	2.100	0.749	0.586
4	*b*	0.545	0.389	0.350	0.020	0.007	0.382
5	*b*	0.465	0.332	0.305	0.026	0.009	0.323
6	*b*	0.470	0.335	0.325	0.045	0.016	0.319
7	*b*	1.190	0.849	0.795	0.045	0.016	0.833

[a] Reduction of oxime-resin with $LiAlH_4$. [b] Reduction by the Leuckart method. [c] Titration by $HClO_4$. [d] Nitrogen contents after treatment with HNO_2. [e] Calculated as the difference between the total and the secondary amine contents.

tanol – water – pyridine – acetic acid 30:24:20:6; S13: 2-propanol – conc. ammonia – water 80:10:10; S14: 1-butanol – acetic acid – water 4:1:1; S20: 2-propanol – water – acetic acid 25:10:1. The chromatograms were visualized by spraying with *t*-butyl hypochlorite followed by tolidin – potassium iodide.

Paper electrophoresis on Whatman No. 1 was conducted at pH 2.2 (acetic acid – water 1:9 v/v) and at pH 6.5 (pyridine – acetic acid – water 9:1:90 v/v) for 3 h at 350 V (LKB Paper Electrophoresis Apparatus).

High performance liquid chromatography. A Spectra-Physics SP-8000 liquid chromatograph with a 10 µl injection loop and a variable wavelength UV-detector operated at 215 or 280 nm was used for HPLC of some peptides. Columns were Licrosorp RP-18, 5 µm from E. Merck and Nucleosil 10 C-18 from Whatman. Mobile phases were gradients between A: 5 mM triethylammonium formate (TEAF) in water and B: 5 mM TEAF in methanol. This buffer is volatile and allows easy isolation of collected samples.

Chromatography on Sephadex-columns. The effluents from the columns were followed by a Uvicord III system (LKB Instruments) equipped with 206 and 280 nm filters.

Materials

The resin materials Bio-Beads S-X1 and S-X2, 200 – 400 mesh were purchased from the Bio-Rad Laboratory. All solvents used were from E. Merck or May and Baker Ltd. Tetrabutylammonium perchlorate was from Fluka AG. Dicyclohexylcarbodiimide from Fluka was distilled *in vacuo*. *p*-Anisoyl chloride (4-methoxybenzoyl chloride) and *p*-toluoyl chloride (4-methylbenzoyl chloride) were of synthetic grade from Aldrich and used without purification. The Boc-amino acids were synthesized in the laboratory according to Schnabel.[24]

As side-chain protecting groups were used: Ztf for Cys, Tyr[25] and Thr,[26] Bzl for Glu and Asp (α-carboxyl group),[27] Mbh for Asn[12] and $BzlNO_2$ for Glu.[27] Methionine was protected as the sulfoxide.[28]

p-*Anisoylpolystyrene – 1 %-divinylbenzene (keto-resin) (1).* Polystyrene – 1 %-divinylbenzene resin (80.0 g) was swelled in nitrobenzene (500 ml) for 2 h. A solution of $AlCl_3$ (20.5 g, 154 mmol) in nitrobenzene (100 ml) and anisoyl chloride (80 ml, 172 mmol) were added at 10 °C. The mixture was stirred for 2 h at 55 °C. The resin was filtered (G-3 glass filter) and washed with dioxan (3 × 200 ml), acetic acid (3 × 200 ml), methanol (3 × 200 ml) and dichloromethane (3 × 200 ml). The resin was then dried *in vacuo* for 2 days at 40 °C. Yield 92.6 g. IR spectrum (KBr) showed a carbonyl-band at 1660 cm^{-1}.

p-*Methoxyphenylketoximylpolystyrene – 1 %-divinylbenzene (oxime-resin) (2).* Keto-resin *1* (20.9 g) was converted to the oxime-resin by suspension in ethanol (300 ml) and pyridine (26 ml) and reaction with hydroxylamine hydrochloride (26.1 g, 376 mmol) overnight at reflux temperature. The resin material was washed with ethanol (3 × 150 ml), ethanol – water (1:1 v/v, 3 × 150 ml), ethanol (3 × 150 ml) and finally dichloromethane (3 × 150 ml). Yield of dried resin 20.8 g. The IR spectrum now showed absence of the 1660 cm^{-1} line.

p-*Anisylaminomethylpolystyrene – 1 %-divinylbenzene (3).* The oxime-resin *2* (20.8 g) was suspended in diethyl ether freshly distilled (o. $LiAlH_4$) and a mixture of $LiAlH_4$ (19.95 g, 526 mmol) in dried ether (1200 ml) was added. The reduction was achieved by reflux at 38 °C overnight. Ethanol (500 ml) was then added followed by addition of hydrochloric acid (3.7 %). The resin was washed on a filter with ethanol (3 × 200 ml), dichloromethane (3 × 200 ml) and 10 % triethylamine in dichloromethane (3 × 200 ml). Yield of dried material 21.8 g. The IR spectrum (KBr) showed strong N – H bands in the region 3400 – 3600 cm^{-1}.

p-*Anisylaminomethylpolystyrene — 1 %-divinylbenzene (4) (H_2N-Bzh(OMe)-S-X1).* Keto-resin *1* (29.3 g) was suspended in nitrobenzene (300 ml) containing ammonium formate (82.5 g) and formamide (100 ml). The flask was fitted with a water trap and heated to 170 °C for 1 h. Formic acid (25 ml) was then added and the addition repeated twice with intervals of 0.5 h. The resin was filtered from the cooled (0 °C) suspension and washed with tetrahydrofuran (375 ml), tetrahydrofuran — ethanol (1:1 v/v, 375 ml) and ethanol (375 ml). The whole procedure was repeated. The resin was then treated with hydrochloric acid (88 ml conc. HCl in 200 ml ethanol) at reflux temperature for 2 h. The hydrolyzed resin *4* was finally washed with portions (125 ml) of ethanol to obtain a filtrate free of chloride. The resin was dried *in vacuo* at 45 °C to yield 29.7 g. Substitution was determined by perchloric acid titration to be 0.35 meqv/g.

p-*Anisoylpolystyrene — 2 %-divinylbenzene (5),* p-*Toluoylpolystyrene — 1 %-divinylbenzene (6),* p-*Toluoylpolystyrene — 2 %-divinylbenzene (7)* were all synthesized analogously with *1*.

p-*Anisylaminomethylpolystyrene — 2 %-divinylbenzene (8) (H_2N-Bzh(OMe)-S-X2),* p-*Tolylaminomethylpolystyrene — 1 %-divinylbenzene (9) (H_2N-Bzh(Me)-S-X1),* p-*Tolylaminomethylpolystyrene — 2 %-divinylbenzene (10) (H_2N-Bzh(Me)-S-X2)* were all synthesized analogously with *4*.

Boc-Val-Met(O)-Thr(Ztf)-Glu(OBzlNO_2)-Ala-NH-Bzh(Me)-S-X1 (11). The synthesis of *11* followed the general scheme. The results from the titrations are shown in Fig. 3a.

Boc-Val-Met(O)-Thr(Ztf)-Glu(OBzlNO_2)-Ala-O-Bzl-S-X2 (12). The synthesis of *12* was carried out in the same way as above. Titration results in Fig. 3b.

Boc-Asn(Mbh)-Tyr(Ztf)-Cys(Ztf)-Asp(α-OBzl)-NH-Bzh(Me)-S-X2 (13). The synthesis of *13* was carried out as above starting from the benzhydrylamine resin *10* and Boc-Asp-α-OBzl. Titration results in Fig. 4a.

Boc-Asn(Mbh)-Tyr(Ztf)-Cys(Ztf)-Asn(Mbh)-O-Bzl-S-X2 (14). The synthesis of *14* followed the general scheme. The starting materials were Boc-Asn(Mbh)-OH as the Cs-salt and the chloromethylated S-X2 resin. The titration results are shown in Fig. 4b.

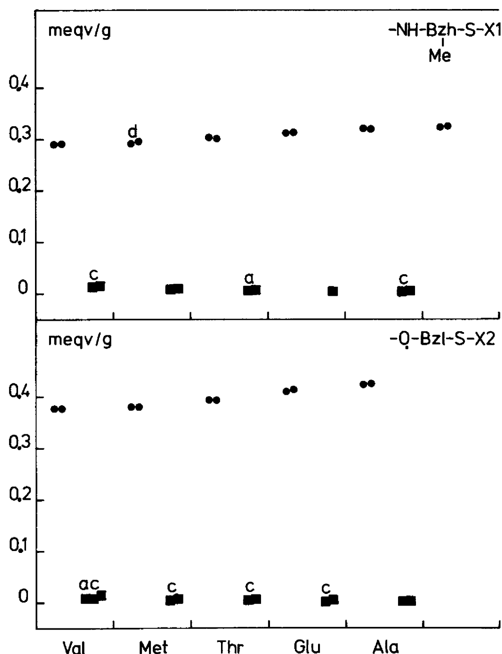


Fig. 3. Synthesis of 11 (3a.) and 12 (3b.) followed by perchloric acid titration. (a) denotes an acetylation step, (c) a repeated coupling, and (d) a repeated deblocking step.

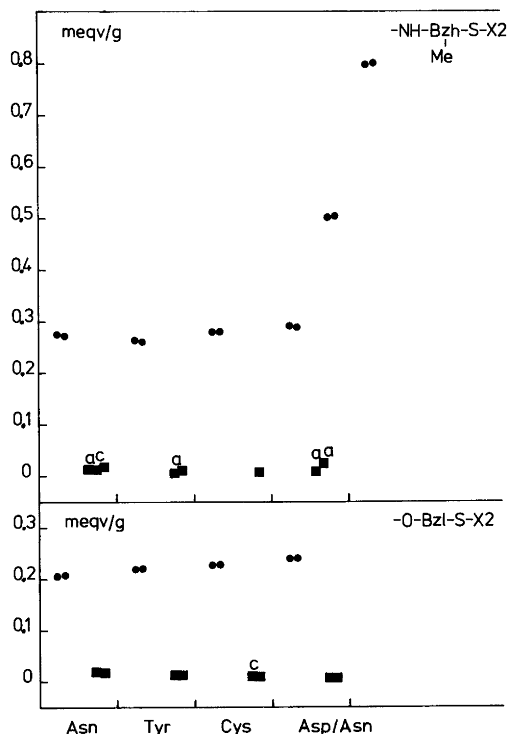


Fig. 4. Synthesis of 13 (4a.) and 14 (4b.) followed by perchloric acid titration. (a) denotes an acetylation step and (c) a repeated coupling.

Val-Met(O)-Thr-Glu-Ala-NH_2 (15). The peptide resin *11* was subjected to cleavage by HBr in trifluoroacetic acid – dichloromethane (1:1 v/v) containing 1 % 1,3-dimethoxybenzene for 2 × 30 min. The combined filtrates were concentrated to an oil and dissolved in *N,N*-dimethylformamide (15 ml). The peptide was precipitated by addition of dry ether (200 ml) with stirring. The precipitation procedure was repeated after conversion of the product to the acetate from the hydrobromide. The product was purified by chromatography on DEAE-cellulose in 25 mM ammonium formate pH 8.5. Tlc (S2, S4, S14, S20; R_f 0.02, 0.26, 0.03; 0.09). Amino acid analysis: $Val_{0.93}Met_{0.83}Thr_{0.93}Glu_{1.00}Ala_{1.00}$.

Paper electrophoresis at pH 2.2 in 10 % acetic acid in water and pH 6.5 in pyridine – acetate buffer showed one band developed by spraying with ninhydrin and fluorescamine. Alanine was used as reference.

Val-Met(O)-Thr-Glu(OBzlNO$_2$)-Ala (16). The peptide resin *12* was subjected to cleavage by HBr and trifluoroacetic acid – dichloromethane (1:1 v/v) as for (11). The peptide was collected as for (15). Purification was performed by chromatography on Sephadex LH-20 using a step gradient from ethanol – water (1:2 v/v) to ethanol – water (7:3 v/v). Tlc (S2, S20; R_f 0.11, 0.23). Amino acid analysis: $Val_{0.95}Met_{0.72}Thr_{0.91}Glu_{1.05}Ala_{1.00}$.

Val-Met(O)-Thr-Glu-Ala (17). The *p*-nitrobenzylester *16* was dissolved in water – ethanol (10:3 v/v, 130 ml) and saponified by addition of 0.1 M NaOH to maintain pH 10.5. The reaction mixture was left at room temperature overnight, neutralized with HCl and evaporated to dryness. The crude material was suspended in water (20 ml) and the supernatant subjected to chromatography on Sephadex LH-20 in water. Tlc (S13, S14, S20; R_f 0.07, 0.22, 0.13). Amino acid analysis: $Val_{1.00}Met_{0.84}Thr_{1.04}Glu_{1.07}Ala_{1.00}$.

Asn-Tyr-Cys-Asn (18). The peptide synthesized on both types of resins *13* and *14* was subjected to 5 M HBr in acetic acid for 5 h. No cleavage could be detected and the products were then cleaved by HBr in trifluoroacetic acid – dichloromethane as mentioned above.

The peptides were then concentrated to an oil, diluted with a small amount of dimethylformamide and extracted repeatedly with light petroleum 60/80 to remove the dimethoxybenzene.

The cleavage mixture contained many components, several of which however did not contain peptide material, and purification could be effected only by HPLC. Both peptides were subjected to repeated runs and the product collected. Amino acid analyses of the products, from resin *13* $Asp_{2.00}Cys_{0.62}Tyr_{0.89}$; from resin *14* $Asp_{2.00}Cys_{0.79}Tyr_{0.75}$.

RESULTS AND DISCUSSION

Peptide synthesis on the benzhydrylamine support monitored by nonaqueous titration with perchloric acid has been shown to be fully possible.

The selection of the resin-type can be based on the stability toward the usual Boc-group deprotecting reagent 1 M HCl in acetic acid, and the lability to cleavage reagents, *e.g.*, 5 M HBr in acetic acid. It has been shown that the stability of the link between the benzhydrylamine resin and the *C*-terminal amino acid toward the deprotecting reagent is very sensitive to changes in the aromaticity of the benzhydryl anchor determined by the substituent (R) (see Fig. 1). The lability toward the cleavage reagents followed the same pattern. The lability increases when a methyl group is exchanged with a methoxy group. Another investigation has shown a correlation between the lability and the amino acid linked to the resin.[29] It is interesting to observe that the lability is crucially different for the single *C*-terminal amino acid and the bonded peptide. In the synthesis of both peptides little or no product was detected when 5 M HBr in acetic acid was used as the cleavage reagent. HBr in trifluoroacetic acid had to be used as the cleavage reagent. This phenomenon can explain low yields in the corresponding HF-cleavage step when using the benzhydrylamine resin without substitution in the aromatic ring. However the reduction step from the keto or oxime resin to the benzhydrylamine resin may contribute to low yields too. Furthermore it can be concluded that the reduction step using metal hydrides as $LiAlH_4$ can result in material with considerable amounts of secondary amine arosen from a base-induced migration of the substituted phenyl group.

In the two-step synthesis of the benzhydrylamine resin it has been difficult to predetermine the extent of final substitution as both reaction steps are sensitive to the reaction conditions used. In the first step it was of practical importance to measure the extent of substitution on the keto resin as a too low substitution could result in the necessity of repeating the first step, and a too high substitution in an incomplete reduction. The Leuckart reduction step (step 2) is very difficult to force to completion. Attempts to measure the content of the resin-bound keto groups in the products of the first step, performed by reduction with $NaBH_4$ followed by iodometric titration failed. The two reaction steps were then followed by IR spectra.

It should be mentioned that several papers describing the syntheses of substituted benzhydrylamine resins state different conditions for the first step.[9,10,29] In this investigation the conditions mentioned in the experimental section were optimum. It can be concluded that the cross-linking parameter did not affect the final substitution but that the extent was higher, a factor of *ca.* 2, for the methyl substitution than for the methoxy with the same reaction conditions.

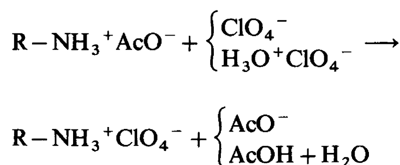
The use of a higher loaded resin did not give any problems in the experiments and followed the general patterns observed. However, to obtain the same degree of substitution of amino acid the first coupling was performed with the amino acid derivative in a calculated deficiency followed by an acetylation of the rest of the amino groups in the resin. All steps were followed by titration with perchloric acid in acetic acid (see Fig. 4a).

The stability experiments show considerably more lability of the bond between the resin and the amino acid during one deblocking cycle for the methoxy resins than for the methyl-substituted resins, as determined by potentiometric titration (see Figs. 2a – c). The cleavage experiments with 5 M HBr in acetic acid show a sufficient lability for all types of resins but somewhat more resistence for the methyl-substituted resins (see Figs. 2d – f). The use of the more acidic reagent HBr in trifluoroacetic acid can be used alternatively. It can be concluded that the methoxy-substituted resins are too labile toward the deprotecting reagent for synthesis of longer peptides and therefore the methyl-substituted resins should be recommended.

The cross-linking parameter has no detectable effect in the stability and cleavage experiments, but no experiment with synthesized peptide resins has been performed. The selection between the two types of supports has been made only in relation to the size of the desired peptides.

However, the extent of cross-linking[31] has a crucial influence on the progress of the potentiometric titration with perchloric acid. The extent of substitution, but not the nature of the substituent, has a similar effect too. It is difficult to understand in detail what is happening in the resin material during the titration, but some characteristic patterns can be observed.

A titration of amino groups in our monitoring method starts with the amino groups in the acetate state. As more titrant is added a net exchange of acetate with perchlorate results. The exchange can follow two of several routes:



$$R-NH_3^+AcO^- + \begin{cases} ClO_4^- \\ H_3O^+ClO_4^- \end{cases} \longrightarrow$$

$$R-NH_3^+ClO_4^- + \begin{cases} AcO^- \\ AcOH + H_2O \end{cases}$$

The electric potential registered in such a titration is determined by the acidic activity outside the resin in the solution. A slow diffusion of molecules in the resin results in potential peaks after every addition of the titrant followed by a slow return of the registered potential before the next addition. A quick diffusion promotes the velocity of exchange of acetate with perchlorate in the resin giving none or smaller peaks and therefore speeds up the titration. This difference, observed for 2 and 1 % cross-linked resins, respectively, confirmed the idea of a more open structure for a low cross-linked resin resulting in quick diffusion of molecules in the resin.

A general decrease in the degree of substitution on the tested resins including the classical Merrifield resin has a similar effect giving the more quick titration pattern (see Fig. 5). Also the ratio of solvent in relation to the amount of resin can affect the titration. More solvent makes the titration slower.

Deviations from the normal progress of a titration have been observed. Continuous clotting of the resin in big lumps and systematically drifting of the electric potential are sometimes noticed. These observations reflect fundamental problems inside the resin. A possible explanation is the special repulsive forces between the nonpolar resin-matrix and the polar growing peptide chain. The phenomenon is especially pronounced when the α-amino groups are in the ionized state as the perchlorate and the low cross-linked resins are used. It should be mentioned that the problems often happen after incorporation of one or several amino acid residues.

More solvent in the starting state of the titration or better addition of an electrolyte *e.g.*, tetrabutylammonium perchlorate eliminates the problem, giving stable potentials at the end of the titration and making the monitoring method applicable. We conclude that the potentiometric titration monitoring method can be extended to these new types of substituted benzhydrylamine resins.

In the synthesis of the pentapeptide on the two different solid supports no significant difference in

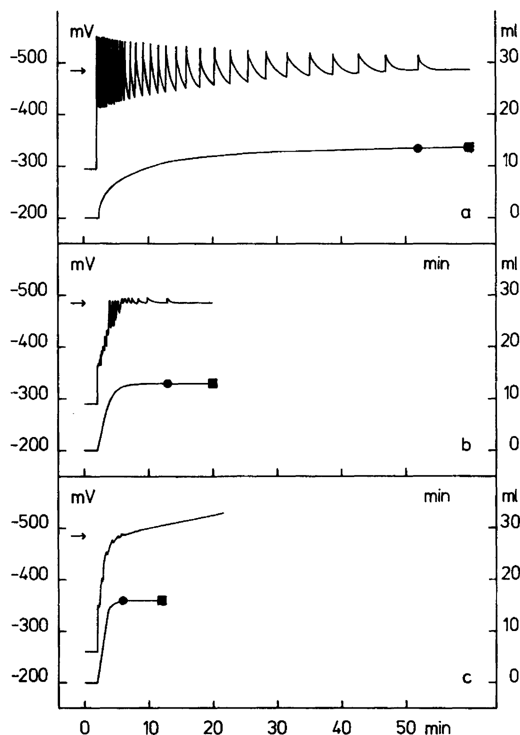


Fig. 5. Typical titration patterns are shown from an S-X2 resin (5a.) an S-X1 resin (5b.) and a resin with drifting potential, usually for the S-X1 resin type only (5c.). ●, Last point of addition of the titrant. ■, Titration shut-off point. →, Set end point of potential. Note that the potential still changes beyond the set end point after shut-off of the titration.

the monitoring results could be observed (see Fig. 3). The peptides synthesized were subjected to cleavage from the resins with HBr in trifluoroacetic acid and purified by usual chromatographic methods.

The tetrapeptide synthesized on the classical Merrifield resin could be purified only on an HPLC-system (see EXPERIMENTAL) and shows several peaks on the chromatogram in accordance with a problematic synthesis as seen from the monitoring scheme (see Fig. 4). The same peptide synthesized on the benzhydrylamine resin was also difficult to purify. However it was considerably more simple to isolate the desired product.

It can be concluded that the substituted type of the benzhydrylamine resin completely can replace the classical Merrifield resins in a normal solid phase procedure including the monitoring method in the synthesis of the peptides mentioned.

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31. The use of Lewis catalysts, *e.g.*, $SnCl_4$ in the chloromethylating step can be of importance as the cross-linking can be increased probably owing to impurities or remains of the cross-linking reagent, *E.g.*, a commercial batch of the chloromethylated resin (S-X2) from Bio-Rad was completely unsuitable to titrate as the titration exceeded 6 h in contrast to our own synthesized batches.

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