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COMPARISON OF THE APPLICABILITY OF 2-HYDROXYETHYLSULFONYLMETHYL- AND CHLOROMETHYL-POLYSTYRENES IN THE SOLID-PHASE SYNTHESIS OF PROTECTED PEPTIDES

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Abstract—In order to compare the applicability of resins in the preparation of protected peptides two solid-phase syntheses of a protected ACTH-(5-10)-hexapeptide have been performed, one using 2-hydroxyethylsulfonylmethyl-polystyrene and the other with Merrifield's chloromethylated polystyrene. To obtain a good comparison, equivalent methods were used as far as possible. Optimal conditions for deprotection of amino groups and for the liberation of the end-product were determined. Chromatographic examination of the crude fission products of the peptidyl resins presented important clues towards the nature of the underlying fission mechanism. Using an automated peptide synthesizer the pure end-product was obtained from both resins in approximately the same yield (*ca.* 70%). It appeared that the isolation of the product from the sulfone resin is less laborious, since the critical alkali-treatment, necessary for the liberation of the product, proceeds faster and, if properly carried out, avoids transesterification.

In the preceding article[1] the preparation of 2-hydroxyethylsulfonylmethyl polystyrene was described and the usefulness of this resin in solid-phase syntheses of peptides was demonstrated by the preparation of a simple model peptide. The resin appeared to provide attractive possibilities for the preparation of protected peptide intermediates, since cleavage from the resin could be performed by a brief treatment with alkali which preserves acid-labile protecting groups. Unexpectedly the carefully chosen alkaline reagent also detached protected peptides from Merrifield's resin during comparable reaction periods. The availability of a common cleavage procedure for adducts of both resins offered an opportunity to compare the general usefulness of the novel sulfone resin with the conventional resin in the preparation of protected peptides. To that aim a protected peptide had to be prepared on each resin, using critically evaluated but generally equal procedures. α-ACTH-(5-10)-hexapeptide (**1a**) in its protected form (**1b**) was chosen as the model-peptide. It contains several amino acids which raise problems in the usual performance of solid-phase syntheses,[2] especially in the acidolytic cleavage of the final peptide-resin ester. Moreover, **1b** constitutes a known intermediate of general suitability[3] for the preparation of corticotrophic peptides.

H-Glu-His-Phe-Arg-Trp-Gly-OH (1a)

Z-Glu(OBu^t)-His-Phe-Arg(NO$_2$)-Trp-Gly-OH (1b)

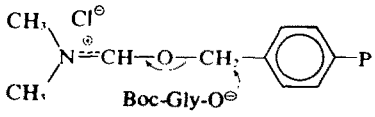
Synthetic design

Boc-groups were used for the temporary protection of α-amino functions, and dicyclohexylcarbodiimide (DCC) as the condensing agent in the successive coupling steps with both resins. In preliminary experiments, Mitin's coupling procedure[4] was attempted for the introduction of the Boc-Arg(NO$_2$) residue. This was in order to circumvent the reported difficulties[5] with this particular amino acid, but the acylation could not be conducted to completion. Eventually the DCC-method was also adopted for the introduction of Arg(NO$_2$). During the last two cycles the side-chain of the histidyl residue was protected with the DNP-group.[6] This protective function is lost during the alkaline detachment of the peptide from the resin, but its selective removal from the resin adduct prior to the detachment, was preferred. This was performed effectively without rupture or derivatisation of the sulfone moiety by thiolysis with thiophenol.[7]

Methods

Introduction of the C-terminal residue into the resins. Esterification of Boc-Gly-OH with the sulfone resin was performed with DCC, as described in the preceding communication. Elemental analysis of resin samples revealed that the esterification rate slowed down with increasing time, probably as a consequence of the varying accessibility of OH groups in the resin. However, from a sulfone resin containing 1.15 mmoles of sulfur/g of resin a useful Boc-Gly-polymer containing 0.34 mmoles of the amino acyl residue/g of resin was obtained in 4 hr. The remaining OH groups were blocked by formylation.[8]

For the introduction of Boc-Gly-OH into the chloromethylated resin the dicyclohexylammonium salt was used. The reaction was performed by suspending the polymer in dimethylformamide and heating the system for several hr at 70° to give a polymeric α-alkoxyimmonium chloride (**2**). This adduct reacted on addition of the dicyclohexylammonium salt with formation of an ester linkage, also yielding dicyclohexylammonium chloride. In



(2)

about 4 hr a resin containing 1.66 mmoles of Cl/g of resin gave a product containing 0.45 mmoles of Boc-Gly-residues/g, but no tertiary amino groups.

Cleavage of Boc-groups. Several acid reagents have been recommended for the removal of Boc-groups from peptides in solution, but not all of them are applicable to the solid-phase method.[9] Special attention was therefore paid to the selection of a reliable cleavage procedure for Boc-groups. Possible damage of the ester bond with the sulfone resin was not contemplated, since experiments with model compounds[10] had revealed that ester bonds in benzylsulfonylethyl amino acid esters are acid stable, and even resistant to 4N HBr in acetic acid. However, it is known that in acid medium sensitive side-chains as present in tryptophan can undergo oxidative deterioration which is usually accompanied by strong discolouration, and alkylation can also occur.

Deprotection of Boc-Trp-OH by dissolution in pure formic acid[11] (6 mmolar) or in 2 N BF_3-etherate[12] in acetic acid (6 mmolar) surpassed cleavage with trifluoroacetic acid–dichloromethane[13,14] or the same reagent in the presence of 10% (v/v) β-mercapto-ethanol. With the above-mentioned reagents deprotection was complete after 10 min. Discolouration was observed only after 4 hr treatment with the second reagent. Derivatisation of liberated tryptophane was not perceptible on TLC.

The trifluoroacetic acid containing reagents required much longer reaction times (about 2.5 hr). In the absence of mercaptoethanol discolouration started in this case after about 1.5 hr. In the presence of the reducing agent colourless by-products were detected after 3 hr.

The usefulness of deprotecting agents for polymeric Boc-derivatives was tested with the Boc-Gly derivative of Merrifield's resin (Table 1). Progress of the cleavage could be followed fairly well from the disappearance of IR-peaks which are due to the Boc-group at 1500, 1385, 1250 and 1050 cm^{-1} or more accurately by TLC of the detached amino acid. No cleavage was observed with formic acid, either concentrated or in the presence of chloroform to swell the resin, Boc-groups were removed easily by the boron trifluoride reagent and the trifluoroacetic acid mixture.

Considering the observations with Boc-Trp-OH in solution the system $BF_3 \cdot OEt_2$/AcOH/HCOOH (0.70 molar) was adopted finally for general use. It combined the reductive properties of formic acid with the favourable characteristics of the boron trifluoride reagent. Two treatments (15 min) were routinely employed. The liberated amino groups were then deprotonated with 10% diethylamine in dichloromethane, which proved to be harmless to the anchoring ester bonds in both resins.

Detachment of the hexapeptide derivative (**1b**). In the preceding article[1] the mixture dioxan/methanol/4N NaOH (30:9:1), being 0.1 N in base (CH_3O^- and OH^-) was selected for cleavage of peptide derivatives from the sulfone resin. In addition to the other reagents mentioned there, solvent mixtures lacking methanol have been examined. Incomplete detachment from a Boc-Gly-resin was observed when methanol was replaced by glycol or glycol monomethyl ether, and when both dioxan and methanol were replaced by sulfolane or hexamethylphosphortriamide. A 0.1 M solution of benzyltrimethylammonium hydroxide in 70% aqueous dioxan only partially removed Boc-Gly-OH during about 18 hr. Tertiary bases (triethylamine, diisopropylethylamine, dicyclohexylethylamine) in dichloromethane or dioxan (up to 10%) failed completely to attack the ester linkage. Reagents containing acetone or dimethoxyethane did not improve the results. Replacement of dioxan by tetrahydrofuran and methanol by 2-propanol resulted in a slowly acting reagent (>30 min). The reported reagent,[15] tetrahydrofuran/methanol/1 N NaOH (7:2:1) which is 0.1 M in base and contains about 10% water could be used, but presumably as a result of limited swelling it acted less rapidly.

In view of the nearly equal acidities of methanol and water the detaching species must be the methoxide ion, which contrary to the hydroxide ion has apparently a good access into the interior of the resin matrix. Consequently, alcohols of lower acidity were unsuitable because their anions do not occur in the presence of water. Anhydrous reagents were not investigated as their higher alkalinities might cause secondary breakdown of the product. Therefore, the original mixture containing methanol and water remained the most suitable.

Table 1. Usefulness of cleavage reagents for Boc-groups in solid-phase synthesis

Deprotecting agent	Molar excess	Deprotection time	Number of treatments	Cleavage of the Boc group
HCOOH 98–100%	510	20 min	1	-
HCOOH 98–100%	1300	20 min	2	-
$HCOOH/CHCl_3$ (1:1)	1300	20 min	2	-
$TFA/CHCl_3$ (1:2)	57	30 min	1	+
$BF_3.OEt_2/CH_3COOH$ (0.33 M)	12.8	20 min	2	+
$TFA/CH_2Cl_2/HCOOH$ (3:6.5:0.5)	53 (TFA)	15 min	2	+
$BF_3.OEt_2$, 0.33 M in $CH_3COOH/HCOOH$ (9:1)	12.8 (BF_3)	20 min	2	+
HCl/THF 1 ml 6N HCl in 14 ml THF	15.4	15 min	1	-

Although basic reagents of comparable alkalinity have successfully been used in classical methods of peptide synthesis, e.g. for the hydrolysis of esters, degradation and racemization might occur as side-reactions. To check this, samples of **1b** prepared by the classical route[3] were subjected to the alkaline environment of the detachment procedure. By-products were formed when the exposure was extended to periods exceeding 3–5 min. This period, however, is more than sufficient for complete liberation of the product. Moreover, degradation of **1b** appeared to occur almost exclusively in solution, and not so long as the peptide derivative remained in the domain of the polymer bead.

Resin-adducts of the Merrifield type were also susceptible to the selected reagent, giving the protected peptide mainly as the free acid. The mechanism is assumed to be a nucleophilic substitution in this case.

The observation that the detaching process from both resins is accompanied by transesterification through methanol was another aspect that had to be considered. In the present case, viz the synthesis of **1b**, the transesterification is not a serious objection, since the methyl ester formed is rapidly hydrolysed on extending the length of the treatment to the maximum permitted period. For general application, however, this transesterification is undesirable. Therefore, the acid/ester ratio in the liberated product was determined by TLC for all the various, intermediate peptides, cleaved from the resins under varying conditions during the complete syntheses (**1b**). Similar products from a classical stepwise synthesis were used as reference compounds. These experiments revealed that the tendency to transesterification diminished with increasing length of the peptide chain.

Very high acid/ester ratios in the products from both resins are always obtained by the general procedure. This consists of a number of treatments with a 15-fold excess of the alkaline reagent at the highest possible agitation rate, each lasting for the maximally allowed period of 3 min. The reaction is terminated by addition of an excess of acetic acid. Under these conditions only traces (<1%) of the methyl ester were detected by TLC in a product which was set free from the sulfone resin in 98% yield within 3 min. Under the same conditions the yield of the product from the Merrifield resin was 71%, and the detachment had to be repeated for a second, third and fourth time to obtain a further 20, 6 and 3%, respectively.

Isolation of the product. For the isolation of the product, filtrates were evaporated *in vacuo*, the residue was dissolved in methanol, and the product was precipitated by addition of water. One crystallization always sufficed to get the pure hexapeptide acid as fine needles, free from its methyl ester and possible degradation products.

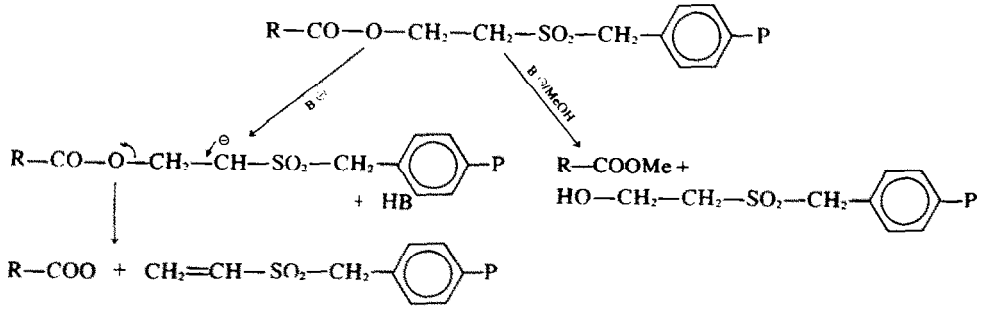
The mechanism of the detachment process. Alkali-induced fission of ester bonds in the prepared resin-adducts can apparently be performed via two different routes; dependent on the site of attack the resulting product is a carboxylate ion or a methyl ester. The preponderance of one of them depends on the relative rates of the triggered reactions which are related to the concentration of the bases in the interior of the resin beads and in the environmental fluid. Liberation of the product from the sulfone resin by β-elimination appeared to be a very rapid reaction. However, it requires a rather high base concentration within the polymer grains to ensure the formation of carbanions from which the expulsion of the carboxylate ion takes place (Scheme 1). Adjustment of a sufficiently high base concentration is counteracted by consumption of base in this reaction. When the required internal base concentration is not reached or maintained, the apparently slower transesterification shows up as a competing reaction. Formation of the carbanion requires a rather hard base which should have easy access to the anchoring bonds: methoxide ions are satisfactory whereas hydroxyl ions are not.

Transesterification of the peptidyl-resin becomes significant when the detachment is performed at low base concentration (e.g. 0.03 M), without agitation of the polymer suspension. When a soft base is used, in combination with anhydrous methanol, the product might well be ejected exclusively in esterified form.[16] Each of these conditions implies an internal base concentration that is too low for pure β-elimination and instead promotes transesterification.

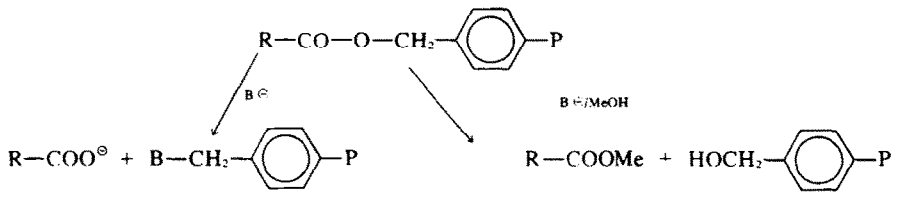
Formally, a similar situation holds for the alkaline detachment, in a resin adduct of the Merrifield type. Here, a base consuming substitution competes with the base catalysed transesterification, but the competition is apparently greater, since crude reaction mixtures contain more of the methyl ester as shown by TLC (Scheme 2). Seeing that more than one treatment is necessary for complete liberation of the carboxylate ion the rate of the substitution reaction is apparently lower than that of the β-elimination from the sulfone resin, but both are considerably more rapid than the transesterification.

RESULTS AND DISCUSSION

The synthesis of the hexapeptide (**1b**) on both resins was performed in about 70 hr using an automated peptide synthesizer and one program. Liberation from the sulfone resin required one treatment with the dioxan/methanol/sodium hydroxide reagent for 3 min to obtain a 66% yield (based on the Boc-Gly content) of the crystalline product, having m.p. 211–215°; $[\alpha]_D - 21.5 \pm 1.0°$ (in DMF). With Merrifield's resin a slightly higher yield was given (77%), but the complete release of the



Scheme 1.



Scheme 2.

product required 3–4 treatments for 3 min each with the basic reagent. The physical constants of the product were the same as for the product from the sulfone resin. The known values[3] are m.p. 210° and $[\alpha]_D - 22.2°$, respectively. Chromatographic examination of both preparations in several solvent systems indicated identity with a classically prepared sample.

From these results it appears that the applicability of the novel sulfone resin as a support in solid-phase syntheses is equally good as that of the approved Merrifield resin. Experimental techniques which have been well tested in peptide syntheses on chloromethylated polystyrenes suffice equally well when applied in a synthesis on the sulfone resin. The new resin may not have evident advantages in syntheses of large, free polypeptides. However, for the preparation of small, protected peptides, intended to be used in further fragment condensations in solution, the sulfone resin may be an attractive, new tool. Liberation of the desired product as a free acid, proceeds faster from this resin than from Merrifield's resin, and is less laborious. Here the risks of racemization or formation of side-products during liberation are smaller.

EXPERIMENTAL

M.ps were determined with a Leitz-Heiztisch Microscope and are uncorrected. Specific rotations were measured with a Perkin-Elmer P141 Polarimeter at 22°. IR spectra were recorded with a Perkin-Elmer 257 spectrophotometer as KBr pellets. For the optimization of the experimental techniques used for cleavage of Boc-groups, for the successive condensation steps, and for the cleavage of the DNP-group, preliminary experiments were performed by a manual method on one or both resins. Any attempted procedure was evaluated chromatographically by comparison of the relevant intermediate after liberation from the resin with the corresponding compound obtained in a classical, stepwise synthesis. In each case methyl esters were included. IR spectroscopy was used also whenever possible. Detachment of such intermediates was performed by suspension of a resin sample in a vigorously stirred, 15-fold excess of the mixture dioxan/methanol/4N NaOH (30:9:1) for 3 min, neutralization of the base by addition of an excess of acetic acid, and centrifugation of the resulting suspension. Chromatographic examination of the clear supernatant was performed in three solvent systems, viz. chloroform/methanol (4:1), n-butanol/acetic acid/water (4:1:1), and n-butanol/acetic acid/pyridine/water (16:1:3:4). The final syntheses were performed in a Schwartz Bio-Research automated Synthesizer loaded with a Boc-Gly polymer which contained 1 mmol of resin-bound glycine.

t-Butyloxycarbonylglycine methylpolystyrene ester. Chloromethylated polystyrene (2 g) containing 5.9% chlorine (1.66 mmoles of Cl/g of resin) was suspended in DMF (10 ml) and left at 70° for 4 hr. Boc-Gly-OH. DCHA (3.32 mmoles) and DMF (5 ml) were added, and the mixture was left at 70° for a further 4.5 hr. During the reaction the resin swelled so strongly that more DMF had to be added. The resulting product was filtered and washed twice with concentrated glacial AcOH (60 ml), successively with 60 ml portions of EtOH, ethanol/water, water, water/methanol and methanol, and finally dried. Elemental analysis gave 0.43% nitrogen, indicating 0.31 mmol of Gly/g of resin.

Table 2. Dcc-Mediated coupling of Boc-amino acids in the automated synthesis of (**1b**)

Step	Reagent	Vol (ml)	Time (min)
1	AcOH	28	5
2	BF_3·OEt_2/AcOH/HCOOH (1:6:5)	28	15,15
3	AcOH (washing)	28	2,2
4	CH_2Cl_2 (washing)	28	5
5	t-BuOH/CH_2Cl_2 (19:1) (washing)	28	5
6	CH_2Cl_2 (washing)	28	5
7	10% D/EA in CH_2Cl_2	28	10,10
8	CH_2Cl_2 (washing)	28	5,5
9	t-BuOH/CH_2Cl_2 (19:1) (washing)	28	5
10	CH_2Cl_2 (washing)	28	5,5
11	2 equivalents of the pertinent Boc-amino acid derivative in the appropriate solvent[a]	13.5	10
12	DCC in CH_2Cl_2 (2 mmoles/6.5 ml)	6.5	b
13	CH_2Cl_2 (washing)	28	5,5
14	EtOH (washing)	28	5,5
15	CH_2Cl_2 (washing)	28	5,5
16-20	as steps 11-15		

[a] In the five subsequent coupling steps Boc-Trp-OH in CH_2Cl_2/DMF (9:1), Boc-Arg(NO_2)-OH in CH_2Cl_2/DMF (5:4), Boc-Phe-OH in CH_2Cl_2, Boc-His(DNP)-OH in CH_2Cl_2/DMF (5:3) and Z-Glu(OBu^t)-OH in CH_2Cl_2, respectively.

[b] Reaction times were 4 and 4 hours for the introduction of the Trp and Arg residues, 4 and 9 hours for Phe and His residues, and 4 and 7 hours for the terminal Glu residue.

t-Butyloxycarbonylglycine 2-ethylsulfonylmethylpolystyrene ester. Hydroxyethylsulfonylmethyl resin[1] (1 g) containing 1.13 mmoles of S/g of resin was suspended in CH_2Cl_2 (5 ml). Boc-Gly-OH (1 g) dissolved in CH_2Cl_2 (10 ml), and DCC (1.17 g) in CH_2Cl_2 (10 ml) were then added to the suspension. The reaction flask was axially rotated for 4 hr. The resulting product was then filtered, washed with three 15 ml portions of CH_2Cl_2, once with AcOH, DMF and EtOH (15 ml each), and finally dried in high *vacuo*. Elemental analysis gave 0.51% nitrogen, indicating 0.36 mmol of Gly/g of resin.

Formylation of residual hydroxyl groups. A formylating reagent was prepared by mixing Ac_2O (0.82 g) and formic acid (0.37 g), heating the mixture for 1 hr at 45°, cooling, and addition of a 1% soln of pyridine in benzene (1 ml). A 10-fold excess of the mixture was added to 1 g of the foregoing resin-adduct. After 19 hr slow agitation the suspension was filtered and washed twice with CH_2Cl_2 (10 ml), with freshly prepared Et_3N/CH_2Cl_2 (1:9) (50 ml), twice with CH_2Cl_2 (10 ml) and with 10 ml portions of EtOH, CH_2Cl_2 and EtOH, and finally dried in high *vacuo*.

Repetitive acylation of the polymeric esters. The scheme for a complete, DCC-mediated coupling cycle of the subsequent amino acid residues is given in Table 2.

Cleavage of DNP-groups from the peptide resins. The resin-peptide adduct (1 g) was treated with 25 ml of thiophenol/DMF (1:9), and the suspension was stirred for 0.5 hr. The yellow-red suspension was then filtered, and the residue was washed three times with CH_2Cl_2 (1.5 ml), then with 1.5 ml portions of DMF, CH_2Cl_2 and MeOH, and finally dried *in vacuo*.

Liberation of the hexapeptide derivative. To a vigorously stirred mixture of dioxan/methanol/4 N NaOH (30:9:1) (25 ml, containing a 15-fold excess of base) an amount of the DNP-free hexapeptide-resin adduct comprising about 0.17 mmoles of the end-product was added. The mixture was agitated vigorously for 3 min, and then treated with an excess of AcOH (0.25 ml). The resin was filtered and washed twice with MeOH (15 ml).

With the Merrifield resin the cleavage procedure was repeated three times. After each treatment the residual resin was washed twice with CH_2Cl_2 (15 ml) and with 15 ml portions of DMF and MeOH. The resin was dried in high *vacuo* before repeating the procedure.

Isolation of Z - Glu(OBu^t) - His - Phe - Arg(NO_2) - Trp - Gly - OH (**1b**). The combined filtrates of the liberation procedure were evaporated. When necessary some toluene was added to remove the last traces of AcOH. The white residue was dissolved in MeOH (10 ml) and crystallization was induced by addition of 30 ml of water. The product was filtered, washed with $MeOH/H_2O$ (1:3), and dried *in vacuo*. A second batch was obtained by evaporation of the mother liquor. It was crystallized as described above. For further purification the product was dissolved in $MeOH/H_2O$ (20:1) and heated to about 70°. Following filtration, water was added at the same temp. until a slight turbidity appeared. On slow cooling the product crystallized as fine glassy needles, m.p. 211–215°, $[\alpha]_D - 21.5°$ ($c = 1$, DMF) from both resins. Found: C, 56.4; H, 6.0; N, 16.2, from the sulfone resin; C, 56.8; H, 6.0; N, 16.0 from the Merrifield resin. Calc. for $C_{51}H_{63}N_{13}O_{13}\cdot AcOH$ (1126.17): C, 56.52; H, 6.0, N, 16.17.

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