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NEW ANCHORS USEFUL IN SOLID PHASE PEPTIDE SYNTHESIS, EVIDENCE OF AN UNEXPECTED POLYACRYLIC SUPPORTED REACTION BY NON DESTRUCTIVE ^{13}C NMR SPECTROSCOPY AND TANDEM MASS SPECTROMETRY

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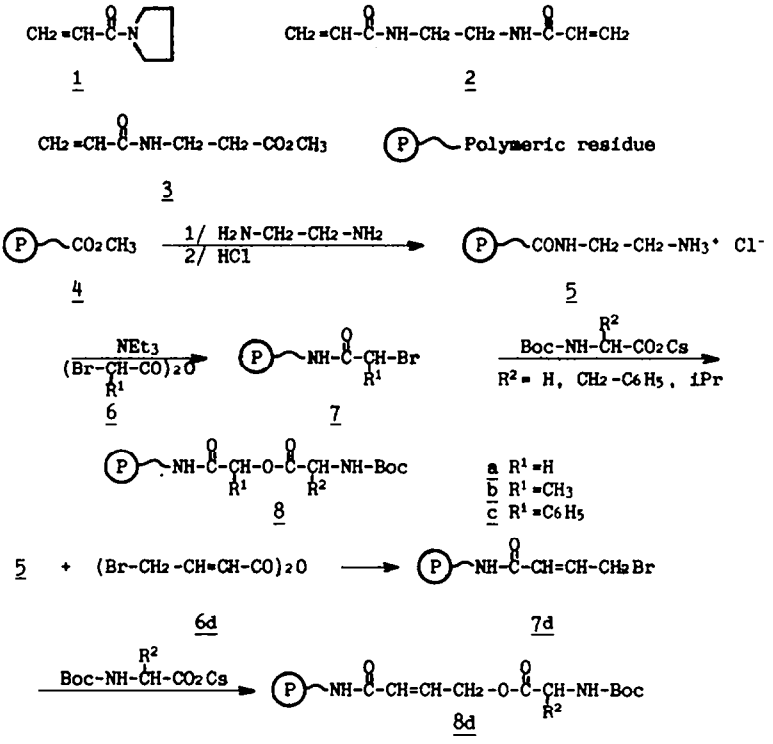
Abstract - A polyacrylic resin with NH2 functions was made to react with α-bromoacetic anhydrides with the view of finding new anchors useful in solid phase peptide synthesis. Besides the normal amide product, the process with α -bromopropionic acid anhydride gave rise to a ß-alanine derivative whose structure was determined by non destructive ^{13}C NMR spectroscopy and tandem mass spectrometry.

Alterations in the normal course of organic reactions by polymer effect are infrequent[1,2]. However, such a case was unexpectedly encountered in our search for new anchors in solid phase peptide synthesis. The preparation of a three dimensional statistical acrylic copolymer intended for peptide synthesis was described in a previous work[3,4,**]. This polymer was obtained in two steps: copolymerisation of three acrylic monomers (N-acryloyl pyrrolidine 1, ethylenebisacrylamide 2 and N-acryloyl methyl ß-alaninate 3) giving 4, then amidification into 5 with ethylenediamine (Scheme 1).

This copolymer 5, functionalized with primary amino groups, was then shown to be an excellent support for solid phase peptide synthesis[5]. In this method the ideal conditions correspond to a completely solvated polymeric backbone, with the peptidic chains completely unfolded and therefore easily accessible to the reagents[6,7]. These favorable conditions were effectively met with polyacrylic supports, but were not completely fulfilled if the first aminoacid is linked to the support by means of an hydrophobic benzyl ester bond, as described in the literature[8-10]. To obtain better results, a new bromoacetic anchor 7a was chosen[5] and obtained by coupling 5 with bromoacetic acid anhydride 6a. The glycolamide bond with the first aminoacid 8a showed good stability against the acidic treatments used in each cycle for cleaving the temporary acido-labile protecting groups of the terminal amine functions. At the end of the synthesis, the glycolamide linkage was quantitatively cleaved in mild non racemizing conditions, this enabling the peptide to be isolated at choice with either a terminal acid, an ester or an amide function[5].

With a view to improving the stability of the ester bond between peptide and support, the bromoacetic anchor has now been replaced by two of its homologues, 7b and 7c respectively, and its 4-bromo crotonic vinylogue 7d. The presence of an additional R^1 group

or a vinyl group can modify both the hydrophobic nature of the anchor and its stability towards hydrolysis.



Scheme 1

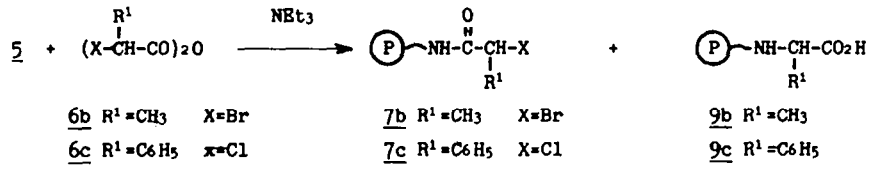
RESULTS

The functionalized polyacrylic support 5[4], containing 0.8 mequiv. of amine function per gramme, was treated according to the described method[5], at room temperature in the presence of triethylamine with a DMF solution containing three equivalents of the α-halogenated acid anhydride 6b, 6c or 6d; Kaiser's ninhydrine qualitative test showed a rapid and complete consumption of the supported amine functions in all three cases. However contrary to the previous case with bromoacetic acid anhydride 6a and the vinylog anhydride 6d, the quantity of bromide ions liberated by hydrolysis of the formed products with a NaOH solution, corresponded to only about 50% of the number of initial amine functions (Table 1).

	halogen %	CO_2H %	bridging %*
6a	100	0	0
6b	55	35	10
6c	52	18	30
6d	100	0	0

Table 1 : Percentages of supported functions proceeding from reaction of 5 with halogeno acid anhydrides 6.
* obtained by difference

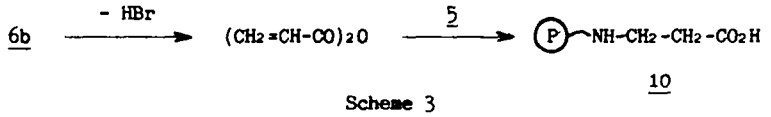
On the other hand carboxylic acid functions appeared and were easily titrated with a NaOH solution. The α-halogenated acid anhydrides 6 being ambident molecules, two competitive reactions may occur with the supported amine 5 : nucleophilic attack of the carbonyl carbon atom with the formation of α-halogenoamide 7, or nucleophilic substitution of the



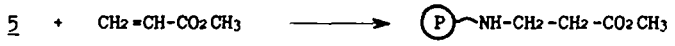
Scheme 2

halogen giving 9 (Scheme 2). An inter-chain reaction between 7 and 5 can also occur, leading to the bridging of two arms. This explains why the sum of the percentage of both the liberated halogens and carboxylic functions is inferior to the percentage of initial amine functions. Similar interchain reactions have already been described [4,11-13]. Thus, replacement of the bromoacetic anchor 7a by 7b or 7c presented some drawbacks which limit their use as reversible anchors. Nevertheless the stability of the corresponding bonds with regard to acidic conditions was studied. Supported derivatives 8b, 8c and 8d were treated for thirty minutes with 30% trifluoroacetic acid in methylene chloride, which are the usual cleavage conditions of the Boc group. An average loss of 0.6% was observed at each cycle, as was the case with the glycolamide linkage[5] 8a.

In the reaction of the supported amine 5 with 6c, the presence of an aromatic ring made the partial substitution reaction of the halogen atom easier. But it is more difficult to understand why 5 reacted differently with the acid anhydrides 6a and 6b. Under these conditions the alanine structure 9b was questioned in favour of the ß-alanine structure 10 which would result from a partial dehydrobromination of 6b followed by an 1,4-addition reaction of 5 (Scheme 3). This type of Michael addition has already been recorded in the reaction of a polystyrene supported amine with an α,ß-ethylenic lactone[14]; it was likewise observed that the treatment of 5 with methyl acrylate led to a support functionalized with carbomethoxy groups with a 82.5% yield (Scheme 4).



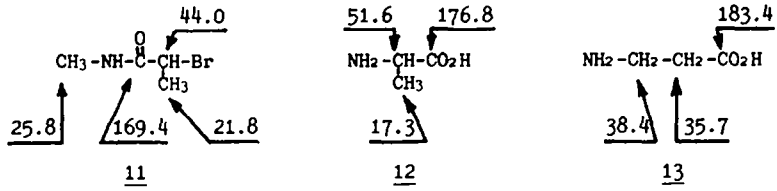
Scheme 3



Scheme 4

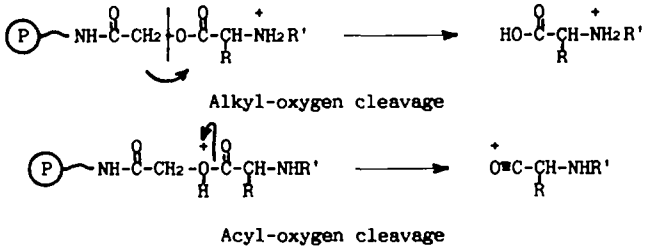
The α-alanine 9b or ß-alanine 10 residue, as well as the accompanying bromoamide derivative 7b, are covalently bonded to the support from which they can neither be cleaved nor structurally studied according to classical methods. Two non-destructive methods were used, thus allowing the direct structural identification of polymer bound entities without preliminary cleavage: ^{13}C NMR[15,16] and mass spectrometry[17].

The ^{13}C NMR spectrum of the supported product was first compared in DMSO-d6 with those of three reference compounds: α-bromo N-methyl propionamide 11, α-alanine 12 and ß-alanine 13[18] (Scheme 5). With the supported product, a single methyl peak at 21.7 ppm was observed, apparently similar to one of the methyl signals of the reference compound 11[18], and presumably corresponding to product 7b. On the other hand, with reference to compound 12[18], no signal appeared around 17.3 ppm that could correspond to the methyl group of a supported α-alanine 9b. However this method did not reveal the presence of the ß-alanine structure 10, the signals of the methylene carbons being masked by those of the polyacrylic support.



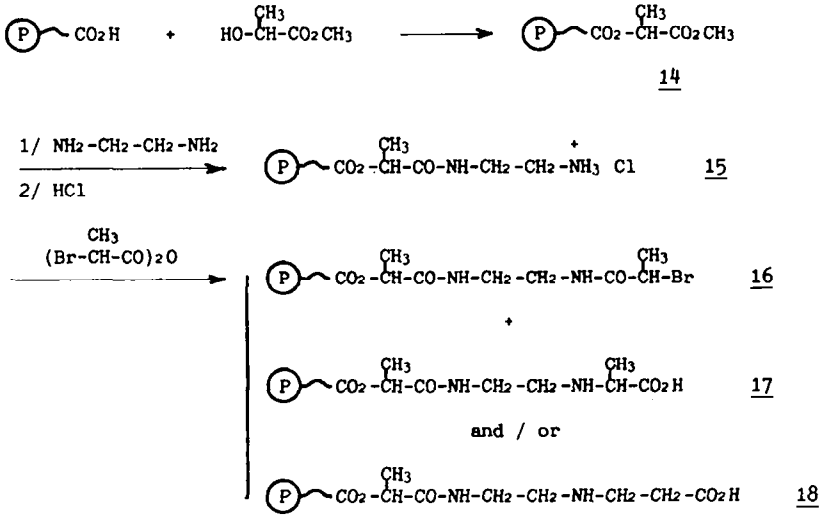
Scheme 5

By means of the FAB (Fast Atom Bombardment) ionisation procedure associated with the MS/MS method (tandem mass spectrometry), it was previously shown[17] for the first time that it is possible to directly analyse the whole system aminoacid-polyacrylic support in a glycerol matrix. This has been applied to the non-destructive monitoring of solid phase peptide synthesis[19]. In the positive mode, the glycolamide bond is cleaved along two different pathways, leaving the polymeric part as a non-charged entity and giving rise to pseudomolecular ions characteristic of the supported peptide[19] (Scheme 6).



Scheme 6

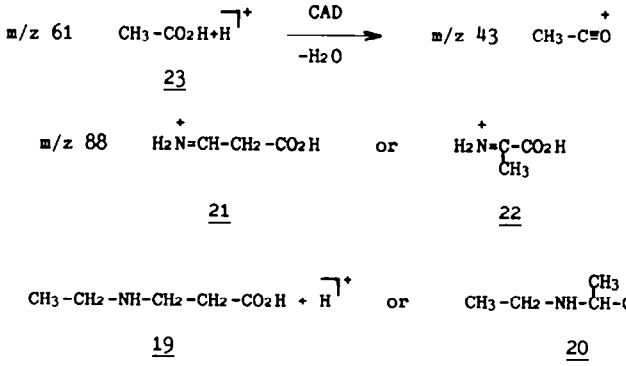
In order to apply this method to the identification of structure 9b or 10, it appeared necessary to establish, between the support and the aminoacid molecule, an ester bond liable to fragment under the conditions of the FAB ionisation method. With this aim, the nature of the previous functionalized arm was modified according to Scheme 7: support 4 was saponified, esterified into 14 with methyl lactate in the presence of DIC and DMAP, then amidified with ethylenediamine into 15. The coupling of 15 with α-bromo propionic acid anhydride was finally carried out under the usual conditions.



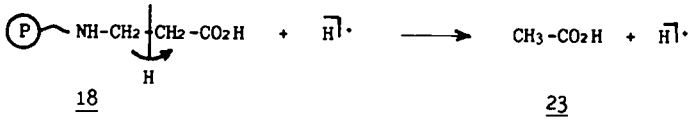
Scheme 7

The percentages of bromide ions liberated by action of a NaOH solution (53%), and of free carboxylic acid functions (36%) were consistent with the previously obtained results in the reaction of 6b or 6c with 5 (Table 1).

The FAB spectrum of this supported mixture of 16 and 17 or 18 was then registered. However the ester bond of 17 or 18 is inverted in comparison with those of Scheme 6, which is liable to modify the fragmentation pattern. Analysis of the sample in a glycerol matrix revealed ions with an even number of electrons characteristic of the functionalized arm (m/z 118, 88 and 61). These ions, for which we propose the following structures (Scheme 8), were observed in the positive mode, beside the characteristic ions of the matrix (m/z 57, 75,93, 167, 185, 277,...). The presence of ion m/z 61 that arises from a protonation followed by a C-C cleavage with the migration of a proton, favours the ß-alanine structure 18 (Scheme 9).



Scheme 8



Scheme 9

The structure of this ion m/z 61 was determined without ambiguity by recording its CAD spectrum (MS/MS technique[20]) which presents (Figure 1) an abundant ion $CH_3-C{\equiv}O^+$ at m/z 43; this ion is formed by the loss of a water molecule, which confirms structure 23. In order to distinguish between structures 21 and 22 (m/z 88), the corresponding CAD spectrum was compared with those obtained with α-alanine and ß-alanine respectively (Scheme 10).

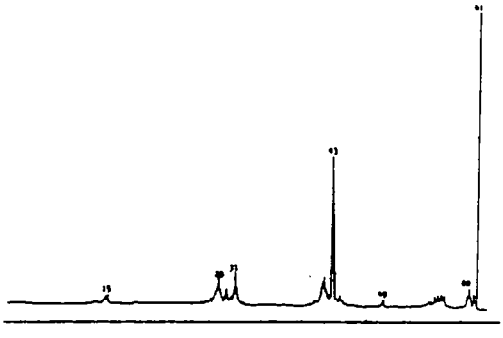
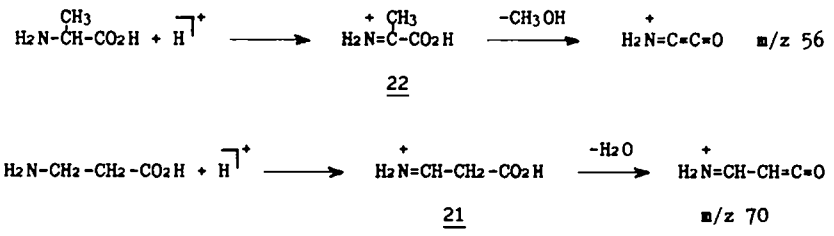


Figure 1



Scheme 10

The CAD spectrum of the ion m/z 88 of α-alanine was characterized by the presence of an ion m/z 56 formed by the loss of methanol. On the contrary, the CAD spectrum of ion m/z 88 from ß-alanine was characterized with a significative abundance by only one ion m/z 70. This ion was formed by the loss of a water molecule from the even electron immonium ion m/z 88. The absence of ion m/z 56 is in accordance with structure 21.

Thus, neither of the two techniques (^{13}C NMR and mass spectrometry) could reveal any evidence of the presence of a supported α-alanine structure. On the other hand, the formation of the ß-alanine structure 18 was demonstrated by mass spectrometry. This last technique therefore appears to be a general and efficient method for the analytical control of supported reactions in non-destructive conditions[17].

The formation of 10 from 6b and of 18 from 15 was unprecedented in homogeneous solutions, where substitutions of α -bromo propionic acid or methyl ester with ammonia and amines are straightforward under the same experimental conditions[21,22]. It was also noted that by treatment with triethylamine alone, α-bromo propionic acid has given a mixture of oligomers probably as a result of a substitution of the bromine atom by the newly formed carboxylate anion. The ^1H NMR spectrum (registered in $CDCl_3$) presented a quartet (at 5.25 ppm) characteristic of a proton bound to the tertiary carbon of a 2-alkyl propionic acid. No triplet corresponding to the CH of a polyacrylic chain and no signal from a vinylic proton could be detected.

EXPERIMENTAL

Abbreviations: Boc, t-butyloxycarbonyl; DCC, dicyclohexylcarbodiimide; DIC, diisopropylcarbodiimide; DIEA, diisopropylethylamine; DMAP, 4-dimethylamino pyridine; DMF, N,N-dimethylformamide; TFA, trifluoroacetic acid.

All ^{13}C NMR spectra of substituted acrylic polymers swollen in DMSO-d6 were recorded with a Brucker WP 200 SY (200 Mhz) spectrometer; mass spectra were recorded with a reversed geometry mass spectrometer using glycerol matrix; the analyser was of an EBE configuration and the gas cell was located before the third analyser[23].

Coupling of α-halogeno acid anhydrides

Acid anhydrides 6 (3 equiv) in DMF (10 ml) and NEt_3 (1 equiv) were successively added under nitrogen to a stirred slurry of polymer 5 (1g, 0.8 mequiv of NH_2) in DMF (10 ml). Stirring was continued at room temperature for two hours (Kaiser's test becomes negative); the resin was filtered and successively washed with DMF, CH_2Cl_2, ethanol, ether and then dried under vacuum in the presence of P_2O_5 at room temperature (α-bromo crotonic anhydride was prepared in the usual way by the reaction of DCC with α-bromo crotonic acid[24]).

Titration of the remaining NH_2 groups

A resin aliquot was washed with a HCl solution (0.1 N), with water until the chloride ions disappeared in the washing solution, with ethanol, ether, then dried under vacuum. Titration is carried out by the Charpentier-Volhard's method[25].

Titration of carboxylic acid functions

A resin aliquot was saponified at room temperature for 4 hours with a NaOH solution (0.5 M), then abundantly washed with water until neutrality, with ethanol followed by ether and finally dried under vacuum at room temperature. Carboxylic acid functions were titrated with a NaOH solution (0.1 N).

Coupling of aminoacids

A slurry of the latter resin (1 g) with the cesium salt of a Boc-aminoacid (5 equiv) in DMF (15 ml) was stirred for 48 hours at 50°C. The resin was then filtered, abundantly washed with DMF, CH_2Cl_2, ether and dried under vacuum at room temperature.

Reaction of 5 with methyl acrylate

A slurry of resin 5 (1 g, 0.8 mequiv NH_2) in DMF (30 ml) containing methyl acrylate (0.2 g, 2.5 equiv) was stirred under nitrogen at room temperature for 12 hours. The resin was filtered and successively washed as above, then dried. After saponification with a NaOH solution (1 M), the liberated carboxylic functions were titrated as described above.

Preparation of resin 16 + 17 and/or 18

After saponification of resin 4 as already described, a product containing 0.8 mequiv of carboxylic acid function per gramme was obtained. To a stirred slurry of 1 g of this material in 15 ml of anhydrous CH_2Cl_2, were successively added a solution of 3 equivalents (0.25 g) of methyl lactate in 10 ml of CH_2Cl_2, 1 equivalent (0,1 g) of DIC and 0.1 equivalent (0.01 g) of DMAP. Stirring was continued for 1 hour at room temperature. After filtering and washing the resin with CH_2Cl_2, a second similar coupling was carried out. Resin 14 was then filtered, abundantly washed as above, and dried under vacuum at 20°C. Titration with a NaOH solution (0.1 N) showed that the initial carboxylic acid functions had disappeared. Resin 15 was now obtained by stirring 14 with ethylenediamine (30 ml/g) for 24 hours at room temperature; after filtration the resin was successively washed with 10% HCl and water until neutrality of the washings, then with ethanol and ether, and dried under vacuum at room temperature. Chloride ions were titrated with the Charpentier-Volhard's method[25]. The final coupling with α-bromo propionic acid anhydride was carried out as described above.

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