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Anchor-linked Intermediates in Peptide Amide Synthesis are Caused by Dimeric Anchors on the Solid Supports.

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Abstract: Cleavage and kinetic studies have been carried out using commercially obtained H-Tyr(*t*Bu)-5-(4′-aminomethyl-3′,5′-dimethoxyphenoxy)valeric acid-TentaGelS (H-Tyr(*t*Bu)-4-ADPV-TentaGelS) and H-Tyr(*t*Bu)-4-ADPV-Ala-aminomethyl-resin (H-Tyr(*t*Bu)-4-ADPV-AM-resin) prepared from commercially available resin and loaded with commercially available Fmoc-4-ADPV-OH amide anchor. Cleavage with pure trifluoroacetic acid (TFA) gave the intermediate H-Tyr-4-ADPV-NH_2, which was then degraded to H-Tyr-NH_2, and cleavage with TFA/dichloromethane (1:9) yielded H-Tyr-4-ADPV-NH_2 which could be isolated in preparative amounts. Cleavage reactions with ^{15}N-labelled H-Ala-4-ADPV-[^{15}N]-Gly-AM-resin yielded the intermediate H-Ala-4-ADPV-NH_2, which contained no ^{15}N as demonstrated by ^1H-NMR. The analysis of the commercial Fmoc-4-ADPV-OH amide anchor showed the presence of Fmoc-4-ADPV-4-ADPV-OH as an impurity in high amounts. This dimeric anchor molecule is the cause of formation of the anchor-linked peptide intermediate obtained during the cleavage from the resin. The particularly high acid-lability of the amide bond between the two ADPV moieties was utilized to synthesize sidechain and C-terminally 4-ADPV protected pentagastrin on a double-anchor resin, and to cleave it using 5% trifluoroacetic acid in dichloromethane. This method may offer a new way for the synthesis of protected peptide amides with improved solubility to be used in fragment condensation.

Keywords: Peptide amide; amide anchors; intermediates in peptide amide synthesis; cleavage of peptide amides

Abbreviations

4-ADPV, 5-(4′-aminomethyl-3′,5′-dimethoxyphenoxy)-valeric acid; AM-PS-DVB, aminomethyl-polystyrene-(1%divinylbenzene)copolymer; DCM, dichloromethane; DIPEA, diisopropylethylamine; DMF, dimethylformamide; Fmoc, fluorenylmethoxycarbonyl; ES-MS, electrospray mass spectrometry; MBHA, methylbenzhydrylamine; MeCN, acetonitrile; TBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3,-tetramethyl-uronium tetrafluoroborate.

INTRODUCTION

Over the last few years a large number of acid-labile anchors [1–6] have been developed for the synthesis of peptide amides by using solid-phase method and Fmoc-strategy. To this list also belongs 4-ADPV anchor published by Albericio and Barany [1] (Figure 1) which is now commercially available. Dürr *et al.* [7] reported in 1991 that by cleavage of H-Ala-4-ADPV-Ala-AM-resin with trifluoroacetic acid/dichloromethane (1:9) the intermediate H-Ala-4-ADPV-NH_2 could be obtained in high yield. By cleaving with pure TFA the same intermediate was formed and then degraded to H-Ala-NH_2 (Figure 2). The formation of H-Ala-4-ADPV-NH_2 was supposed to arise by the cleavage of the NH–CH_α bond of the alanyl spacer. In response to this finding Albericio and Barany [8] claimed that they could not reproduce the same result by cleaving Fmoc-4-ADPV-Ala-Nle-AM-resin

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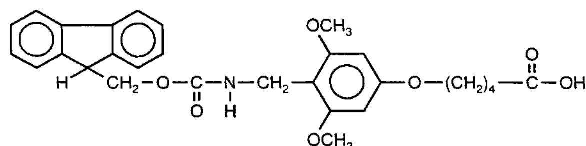


Figure 1. Structural formula of the amide anchor 5-[4'-(fluorenylmethoxycarbonyl)-aminomethyl-3',5'- dimethoxy-phenoxy]valeric acid (4-ADPV).

and Fmoc-Val-4-ADPV-Ala-Nle-AM-resin under the conditions used by Dürr *et al.* [7], since they obtained Fmoc-NH_2 and Fmoc-Val-NH_2 as the sole cleavage products, and amino acid analysis of the resin before and after the cleavage gave the same loading values for the spacer amino acids Ala and Nle.

The aim of the present publication is to produce a final clarification on the possible cleavage sites of 4-ADPV-resins. For this purpose cleavage reactions have been carried out on commercially purchased 4-ADPV-TentaGelS-resin and on aminomethylated polystyrene resin loaded with commercially available Fmoc-4-ADPV-OH amide anchor. In addition we present a procedure for the preparative synthesis of sidechain and C-terminally 4-ADPV-protected peptide amides, which exhibit enhanced solubility.

MATERIALS AND METHODS

Materials

As starting materials for the synthesis of Tyr-4-ADPV-resins the following compounds have been employed: aminomethyl-polystyrene(1% divinylbenzene) copolymer (AM-PS-DVB) with a loading of 1.3 mmol/g as well as Fmoc-Tyr(*t*Bu)-4-ADPV-TentaGelS with a loading of 0.23 mmol/g were purchased from Rapp Polymere, Tübingen. Fmoc-4-ADPV-OH was purchased from Serva (Heidelberg). Fmoc-amino acids were obtained from Orpegen (Heidelberg) and Novabiochem (Läufelfingen), pentagastrin was purchased from Novabiochem, ^{15}N-glycine was obtained from Promochem (Wesel) and derivatized by using Fmoc-OSu [9].

Synthesis

Coupling of the spacer Fmoc-Ala-OH, Fmoc-4-ADPV-OH anchor and Fmoc-Tyr(*t*Bu)-OH to the amino-methyl-resin was carried out with 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate, 1-hydroxybenzotriazole and diisopropylethylamine in dimethylformamide. That the reactions were complete was verified with the Kaiser test [10], the unreacted free amino groups were acetylated with

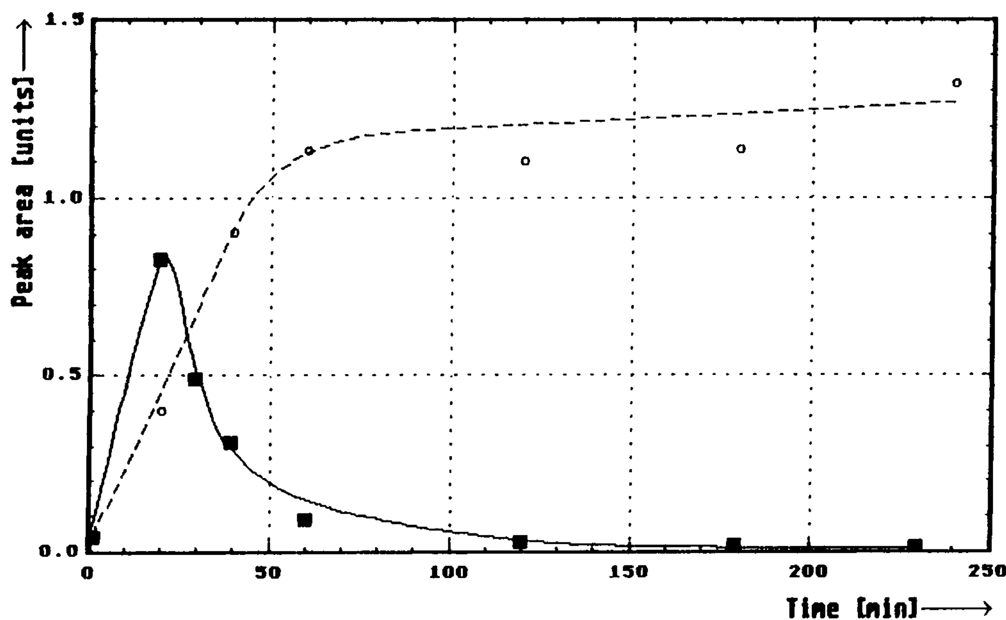


Figure 2. Kinetics of the intermediate H-Ala-4-ADPV-NH_2 during the cleavage from H-Ala-4-ADPV-Ala-AM-resin: (■) 100% TFA; (○) 10% TFA (7).

acetic anhydride and diisopropylethylamine. Cleavage of the Fmoc-protecting group was effected with piperidine/dimethylformamide (1:1, v/v) in 20 min.

All the peptide–resin cleaving reactions were carried out at room temperature by treating the peptide–resins with different mixtures of trifluoroacetic acid/dichloromethane while stirring. The samples taken from the reaction mixtures (150 μl) were immediately mixed with water/acetonitrile (2:1, v/v; 100 μl) and the polymeric materials were removed by centrifugation.

Analytic Methods

For identification of the cleavage products a Waters HPLC system (Eschborn) consisting of multisolvent delivery system 600, WISP autosampler 712 and photodiode array detector 990 was employed. Analytical separations were carried out on a Nucleosil C-18 column (5 μm, 2 × 250 mm, Grom, Ammerbuch) with a flow rate of 0.3 ml/min. For semi-preparative HPLC runs a Nucleosil C-18 column (7μ, 10 × 250 mm, Grom, Ammerbuch) with a flow rate of 3.5 ml/min was used. Water and acetonitrile (MeCN) containing 0.1% TFA were used as solvents. The following linear gradient systems were used for the separation: A, 10–60% MeCN within 30 min; B, 30–70% MeCN within 20 min; C, 50–100% MeCN within 30 min. The final concentration of MeCN was kept isocratically for 5 min. Thin layer chromatography (TLC) was performed on precoated plates of silica gel 60 F_{254} (Merck, Darmstadt), using chloroform–methanol–water (85:15:2) as solvent system.

Characterization of the products

Electrospray mass spectra (ES-MS) and tandem mass spectra (MS-MS) were taken on a API III triple-quadrupole mass spectrometer (Sciex, Thornhill, Canada) by injecting the peaks collected from the analytical and semipreparative HPLC. On-line HPLC-mass spectrometry (LC-MS) was carried out by direct injection of the eluate obtained from a Nucleosil C-18 column (5μ, 2 × 250 mm, Grom, Ammerbuch) with a flow rate of 0.3 ml/min (gradient C) and simultaneous UV detection at 254 nm.

High-resolution NMR spectra were recorded on a Bruker AMX 600 spectrometer (Bruker Physics, Karlsruhe, Germany) using an inverse triple resonance probe. 2.8–9.2 mM solutions in deuterated DMSO were used. All two-dimensional spectra were acquired at 305 K in the phase-sensitive mode. Quadrature detection in F1 dimensions was obtained by using the TPPI method. Generally 512 experiments with 64–96 scans and data size of 2 K complex points were collected for each of the two-dimensional experiments. The TOCSY experiments consisted of a MLEV 17-spin lock sequence with a mixing time of 70 ms. For the ROESY experiment a cw spin lock with 300 ms mixing time was used. The HMQC contained a BIRD pulse sequence to suppress signals of protons bound to ^{12}C. Data processing consisted of zerofilling to 4 K (F2) and to 1 K in the F1 dimension and apodisation with a shifted squared sine-bell weighting function.

RESULTS AND DISCUSSION

In the first series of experiments the two cleavage behaviours of the two different resins containing Fmoc-Tyr(*t*Bu) linked via the 4-ADPV anchor were compared. For this purpose the Fmoc-protecting group of the purchased Fmoc-Tyr(*t*Bu)-4-ADPV-TentaGelS-resin was cleaved. Moreover, H-Tyr(*t*Bu)-4-ADPV-Ala-AM-PS-DVB was synthesized on the aminomethylated polystyrene resin using Fmoc-strategy and the commercially obtained Fmoc-4-ADPV-OH linker. For the cleavage experiments, samples of both resins (20 mg) were treated with 1 ml of the cleavage solution **1** (Table I). HPLC peaks obtained from the two cleavage solutions (Figure 3) were collected and identified by means of ES-MS as: **A** (Retention Time, RT = 3.5 min): H-Tyr-NH_2 (this compound was co-eluted with the injection peak) and **B** (RT = 18.1 min): H-Tyr-4-ADPV-NH_2.

In order to investigate the cleavage kinetics on both resins, each amino acid loaded resin was treated according to experiments **2a** and **2b** (Table I). The results confirmed unequivocally the initial formation and subsequent degradation of H-Tyr-4-ADPV-NH_2 according to the findings of Dürr *et al.* [7] (Figure 2).

In order to investigate the assumption of a NH-CH_α cleavage made by Dürr *et al.* [7] more deeply, H-Ala-4-ADPV-[^{15}N]-Gly-AM-resin was synthesized by using commercially obtained Fmoc-4-ADPV-OH linker and fully labelled ^{15}N-glycine as a spacer amino acid. This resin (500 mg) was cleaved with TFA (10 ml) for 20 min, mixed with water (10 ml) and filtered off. The solution was concentrated *in vacuo*. Separation and purification of the obtained H-Ala-4-ADPV-amide was achieved by semipreparative HPLC. The ^{1}H-NMR spectrum of this anchor-linked alanine amide is identical with or without ^{15}N-decoupling, i.e. the presence of ^{15}N in the valeric acid amide group is ruled out. An NH–CH_α bond cleavage of the amino

Table I Experimental Conditions for Cleavage of Peptide-Resins[a]

Experiment	Cleavage Solution	Volume (ml)	Time(min)
1	TFA	1	20
2a	TFA/DCM (1:9)	2	180[b]
2b	TFA	2	180[b]
3	TFA/DCM (1:99)	1	40[c]
4a	TFA/Scavenger[d]	1.2	120
4b	TFA/DCM (0.1:99.9)	2 × 1[e]	120
4c	TFA/DCM (5:95)	2 × 1[e]	120

[a] Experiments **1**, **2a** and **2b** refer to the cleavage from H-Tyr(tBu)-4-ADPV-TentaGelS-resin and H-Tyr(tBu)-4-ADPV-Ala-AM-resin. Experiment **3** refers to the cleavage from H-Gly-Trp(Boc)-Met-Asp(tBu)-Phe-ADPV-ADPV-Ala-AM-resin. Experiment **5** describes the cleavage of the peptide mixture present in **4c**. For each experiment the amount of resin sample was 20 mg.
[b] Samples for analysis (150 µl) were taken after 10, 20, 30, 40, 50, 60, 90, 120, 150 and 180 min.
[c] Samples for analysis (150 µl) were taken after 1, 3, 5, 10, 20, 30 and 40 min.
[d] Reagent K (82.5% TFA, 5% phenol, 5% thioanisole, 2.5% 1,2-ethanedithiol, 5% water) was used as a scavenger.
[e] Cleavage mixture was filtered after 1 h of reaction time and the cleavage was continued for another hour using a fresh solution.

acid spacer does not occur under the applied cleavage conditions.

In order to have an explanation for the formation of 4-ADPV intermediate, several further experiments have been carried out. First of all it had to be clarified whether the Fmoc-protecting group of Fmoc-4-ADPV-OH may have been lost under the coupling conditions (TBTU, HOBt, DIPEA in DMF). Such a deprotection has already been observed during the coupling of Fmoc-Gly-OH [11]. The tertiary amine DIPEA effects 1–2% cleavage of the Fmoc-protecting group and therefore leads to Gly-Gly double couplings. A doubly coupled 4-ADPV anchor would lead to the formation of the 4-ADPV intermediate observed during the cleavage experiment (Figure 4).

The behaviour of Fmoc-4-ADPV-OH under the coupling conditions was investigated in DMF solution after addition of TBTU, HOBt and DIPEA in the molar ratio of 1:1:1:1.5. These conditions are exactly the same as applied in the case of a 'real' coupling. The mixture was then shaken for 2 h and analyzed by TLC. By comparing the results of TLC with those of the freshly prepared Fmoc-4-ADPV-OH (RF = 0.71) and 4-ADPV-OH (this free anchor would have been formed by Fmoc cleavage, RF = 0.06) no change of Fmoc-4-ADPV-OH present in the coupling mixture could be observed.

In another experiment Fmoc-4-ADPV-Ala-AM-resin was treated exactly as in the previous experiment. At the end of the reaction time the resin was filtered off and the filtrate was analyzed by HPLC. In this case only the components present in the reaction mixture,

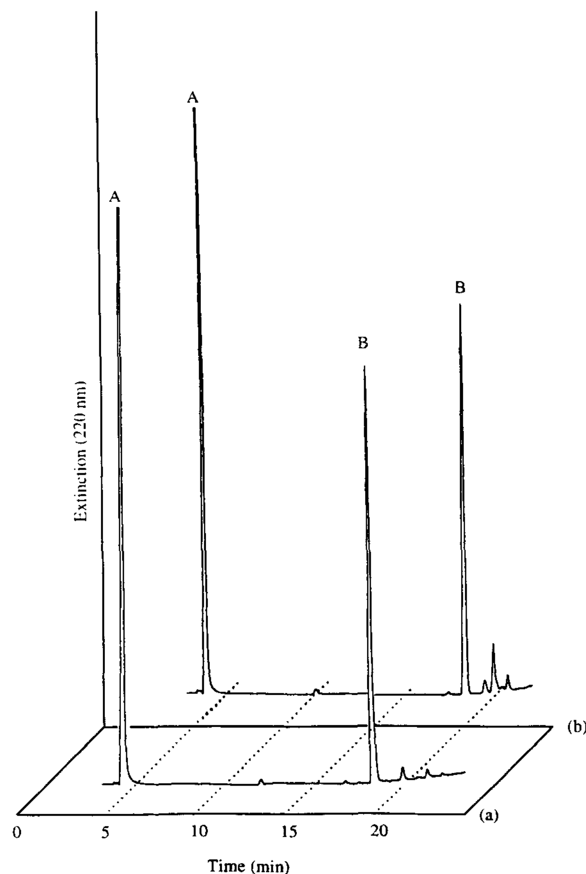


Figure 3. HPLC chromatogram (gradient A) of the cleavage products from (a) H-Tyr(tBu)-4-ADPV-TentaGelS-resin and (b) H-Tyr(tBu)-4-ADPV-Ala-AM-resin with 100% TFA, 20 min, according to **1** (Table I): **A**, H-Tyr-NH_2 (the compound co-elutes with the injection peak); **B**, H-Tyr-4-ADPV-NH_2.

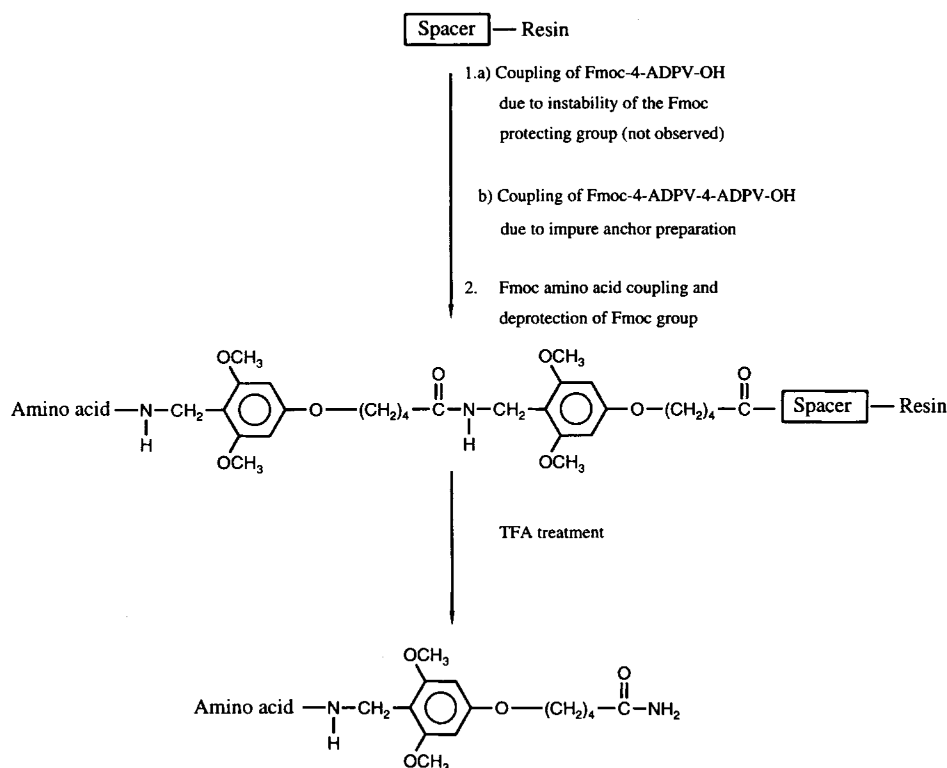


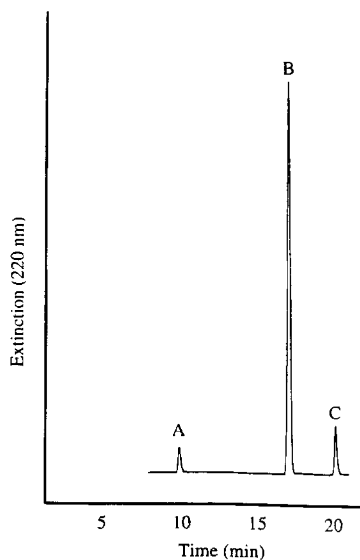
Figure 4. Formation of anchor dimers on the resins with the highly acid-sensitive cleavage site.

i.e. TBTU, HOBt, DMF and DIPEA, could be found. Determination of the load on the resin before and after the experiment gave the same results. Therefore it could be concluded that the Fmoc group of Fmoc-4-ADPV-OH shows no tendency to be cleaved under the coupling conditions. Double couplings to form 4-ADPV-4-ADPV are thus ruled out.

Since cleavage of the Fmoc group under conditions of the coupling reaction was not observed, the commercially obtained Fmoc-4-ADPV-OH was subjected to an exact analysis. Unexpectedly the HPLC chromatogram of this compound (Figure 5) showed the presence of three peaks which have been identified by means of on-line LC-MS as Fmoc-4-ADPV-NH_2 (RT = 12.5 min), Fmoc-4-ADPV-OH (RT = 16.8 min) and Fmoc-4-ADPV-4-ADPV-OH (RT = 20 min). The compound Fmoc-4-ADPV-NH_2 could only be found in the LC mass spectrum but not in the ES-mass spectrum. Owing to this fact and to the extreme acid-lability of Fmoc-4-ADPV-4-ADPV-OH (see below) we suppose that the TFA in the HPLC solutions is responsible for the formation of Fmoc-4-ADPV-NH_2 by cleaving Fmoc-4-ADPV-4-ADPV-OH. Commercially obtained Fmoc-4-ADPV-OH (13 mg, 0.026 mmol) containing the ADPV dimer as an impurity was treated with 0.1% TFA according to **3**. By this reaction Fmoc-4-ADPV-NH_2 was primarily formed which was degraded to Fmoc-NH_2 during the course of the reaction. This facile degradation is remarkable since tyrosine amide could not be observed in the case of H-Tyr-4-ADPV-NH_2 by treatment with a more concentrated TFA solution (10%). Obviously the cleavage site between the Fmoc group and 4-ADPV anchor is more acid-labile than the cleavage site between the amino acid and 4-ADPV anchor.

The extreme acid-labiliy of Fmoc-4-ADPV-4-ADPV-OH led to the idea that by cleaving a resin such as H-peptide(protected)-ADPV-ADPV-spacer-AM-resin with a highly diluted TFA solution, the C-terminally protected peptide amide H-peptide(protected)-ADPV-NH_2 could be obtained. Owing to their improved solubility such amide-protected peptides could be used, e.g. for fragment condensations. Cleavage of the C-terminal ADPV protecting group and all sidechain protecting groups could then be achieved with concentrated TFA. Synthesis of the double-anchor resin was carried out manually as described under Materials and Methods, synthesis of the protected peptide amide pentagastrin H-Gly-Trp(Boc)-Met-Asp(*t*Bu)-Phe-NH_2 was achieved by coupling Fmoc-amino acids with TBTU/HOBt/DIPEA using an ABI 430 A peptide synthesizer.

At first a small amount of peptide-resin (20 mg)



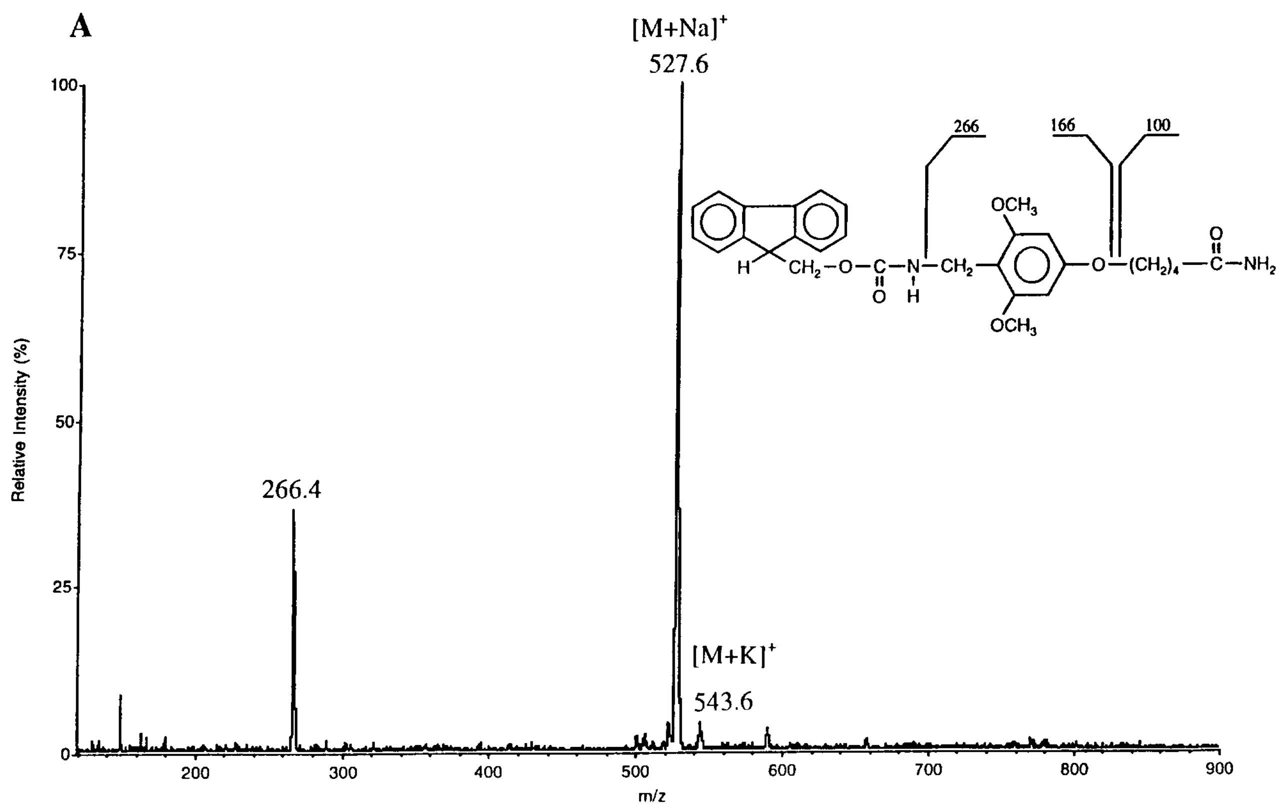


Figure 5. HPLC chromatogram (gradient C) and LC-MS spectra of commercially purchased Fmoc-4-ADPV-OH: **A**, Fmoc-4-ADPV-NH_2; **B**, Fmoc-4-ADPV-OH; **C**, Fmoc-4-ADPV-4-ADPV-OH.

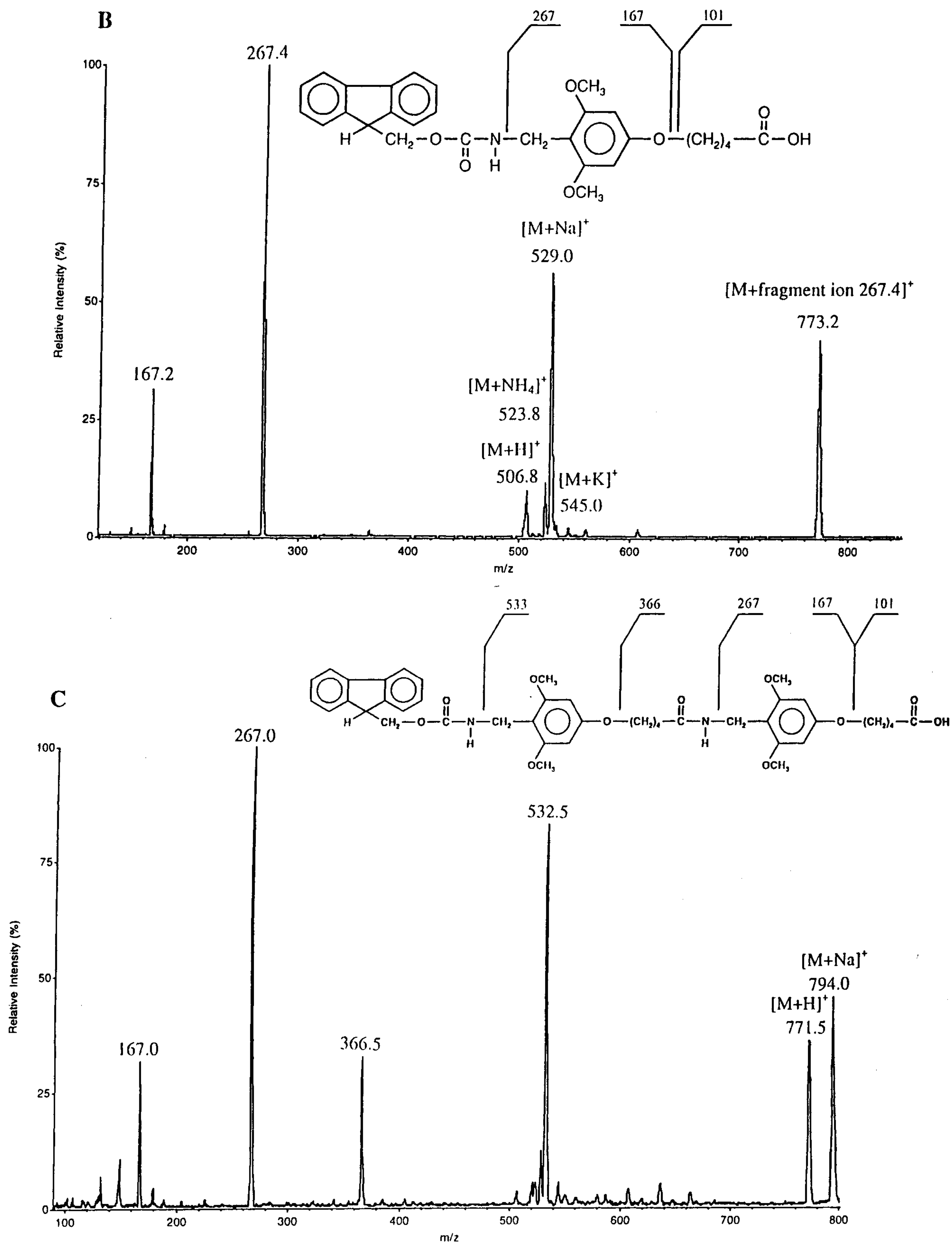


Figure 5. Continued.

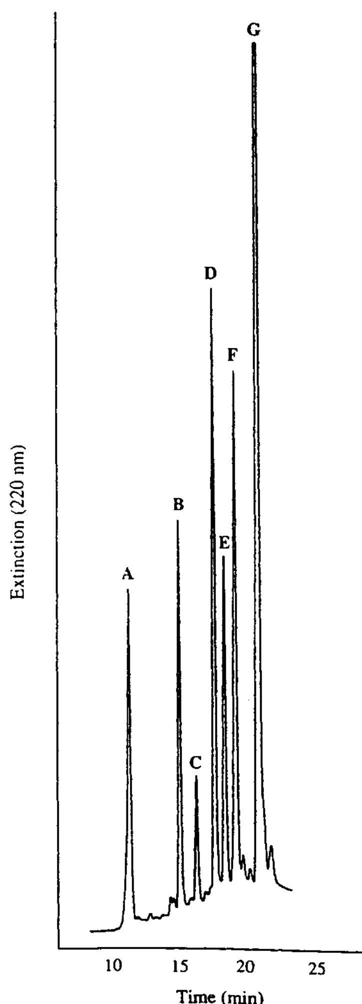


Figure 6. HPLC chromatogram (gradient B) of the product mixture obtained after cleavage from pentagastrin-4-ADPV-4-ADPV-Ala-AM-resin with 5% TFA according to **4c** (Table I). Peak assignment was done by ES-MS (A to G) and 2D ^{1}H-NMR (G).

was cleaved according to **4a** to obtain pentagastrin H-Gly-Trp-Met-Asp-Phe-NH_2. The prepared peptide was analytically compared with a sample of the commercially available pentagastrin by HPLC and ES-MS. Cleavage of a second peptide-polymer sample (20 mg) with 1% TFA solution according to **4b** yielded no products. Cleavage of the peptide from the resin could be achieved only after increasing the TFA concentration to 5%, **4c**. The HPLC chromatogram of **4c** revealed the presence of seven peaks (Figure 6), which were identified by ES-MS to be as follows:

A (RT = 11.5 min): H-Gly-Trp-Met-Asp-Phe-NH_2
B (RT = 15.5 min): H-Gly-Trp-Met-Asp-Phe-4-ADPV-NH_2
C (RT = 16.5 min): H-Gly-Trp(Boc)-Met-Asp-Phe-NH_2
D (RT = 17.5 min): H-Gly-Trp-Met-Asp(*t*Bu)-Phe-4-ADPV-NH_2
E (RT = 18.2 min): H-Gly-Trp(Boc)-Met-Asp-Phe-4-ADPV-NH_2
F (RT = 19.2 min): H-Gly-Trp(Boc)-Met-Asp(*t*Bu)-Phe-NH_2
G (RT = 21.0 min): H-Gly-Trp(Boc)-Met-Asp(*t*Bu)-Phe-4-ADPV-NH_2

The sidechain and C-terminally 4-ADPV protected product G was isolated from the mixture of semi-preparative HPLC and analysed by ^{1}H-NMR spectroscopy. Complete assignment of all ^{1}H as well as all the ^{13}C resonances (except Cq) was possible by using two-dimensional techniques (TOCSY, ROESY, HMQC). A series of ROE contacts between Phe and 4-ADPV signals clearly demonstrated the C-terminal position of the ADPV group (Figure 7). Therefore, alkylation of Trp by ADPV, observed by Albericio *et al.*

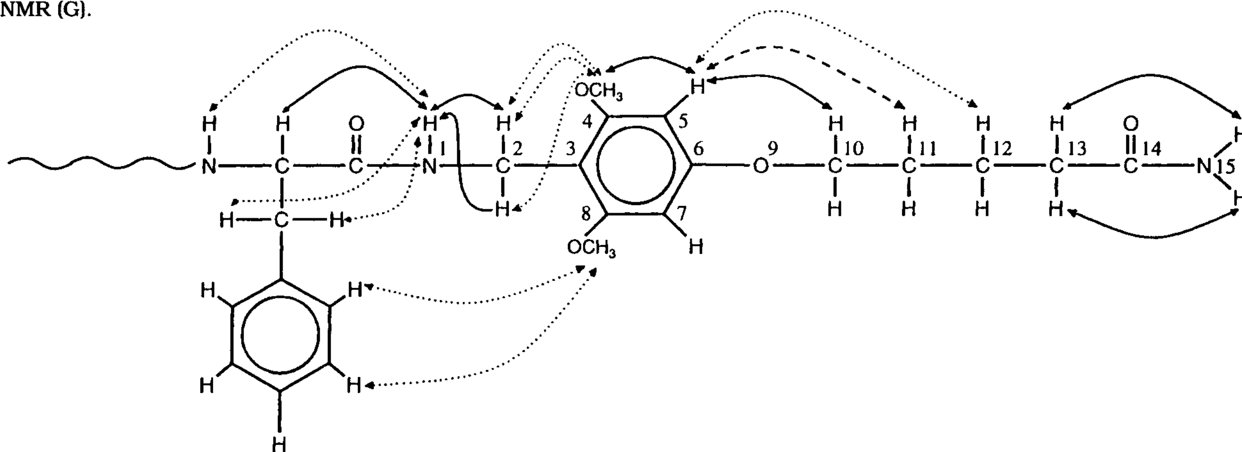


Figure 7. C-terminal part of pentagastrin: ROE contacts between Phe and 4-ADPV signals. ⟷ strong, ← – – → medium, ← · · · → weak.

Table II ^1H Chemical Shifts of Pentagastrin-4-ADPV-NH_2

Residue	Chemical shift (ppm) for			
	NH	H_α	H_β	others
Gly	7.86	3. 36, 3.55		
Trp(Boc)	8.60	4.47	2.81, 309	7.53, 7.99, 7.23, 7.74, 7.30 (aromatic) 1.61 (Boc)
Met	8.44	4.35	1.77, 1.90	2.40 (γ), 2.01 (CH_3)
Asp(tBu)	8.16	4.54	2.39, 2.60	1.33 (tBu)
Phe	7.72	4.46	2.75, 2.88	7.16, 7.13, 7.19 (aromatic)
ADPV[a]	7.55 (1); 4.16, 4.09 (2); 3.72 (4, 8); 6.20 (5, 7); 3.91 (10); 1.68 (11); 1.62 (12); 2.10 (13); 6.66, 7.21 (15)			

[a] Numbering of 4-ADPV according to Figure 7.

Table III ^{13}C Chemical Shifts of Pentagastrin-4-ADPV-NH_2

Residue	Chemical shift (ppm) for			
	C_α	C_β	C_γ	others
Gly	39.8			
Trp(Boc)	52 .1	27.2		123.9, 114.1, 121.9, 119.0, 123.9 (aromatic) 27.4 (Boc)
Met	51.5	31.9	29.2	14.3 (CH_3)
Asp(tBu)	49.1	37.0		27.3 (tBu)
Phe	53.2	37.7		127.4, 128.7, 125.7 (aromatic)
ADPV[a]	31.3 (2); 55.4 (4, 8); 90.8 (5, 7); 61.0 (10); 28.0 (11); 21.4 (12); 34.3 (13)			

[a] Numbering of 4-ADPV anchor according to Figure 7.

[12] during the cleavage of Trp-containing peptides from 4-ADPV-MBHA-resins and also by Dürr *et al.* [7], does not occur during this cleavage of pentagastrin (Tables II and III).

Integrating the HPLC peak areas (Figure 6) and assuming similar molar extinction coefficients, the percentage of the desired fully protected peptide amide G in the cleavage mixture was judged to be about 27%. A repetition of the cleavage of the raw product according to **4a** (Table I) yielded 73% of free pentagastrin.

CONCLUSIONS

The intermediates formed during cleavage of the commercially available 4-ADPV-resins and 4-ADPV-linker-resins are due to the presence of dimeric ADPV in these solid supports, since a double coupling of the anchor through cleavage of a labile Fmoc protecting group as well as cleavage of the NH–CH_α bond of the amino acid spacer could definitively be ruled out. Needless to say that impurities not only in protected amino acids but also in commercial compounds used for their anchoring may be a source of by-products in peptide synthesis.

We were successful in synthesizing a sidechain and C-terminally 4-ADPV protected peptide. Unfortunately the cleavage from the double anchor resin could not be achieved with 1% TFA solution. The result of the use of a higher concentrated TFA solution was a mixture of differently protected pentagastrin molecules and therefore we could isolate the desired peptide only in low yield. Assessment

should take into account that this was a non-optimized synthesis.

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