SOLID PHASE SYNTHESIS OF A TETRAPEPTIDE LABELLED WITH CARBON-13

Preparation of L-[U-^{13}C]Lysyl-L-[1-^{13}C]Arginyl-L-[3-^{13}C]Aspartyl-L-[1-^{13}C]Serine

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SUMMARY

Synthesis of a [^{13}C] tetrapeptide L-[U-^{13}C]Lysyl-L-[1-^{13}C]Arginyl-L-[3-^{13}C]Aspartyl-L-[1-^{13}C] Serine (KRDS) on a *p*-alkoxybenzyl ester polystyrene-1% divinylbenzene resin support is described by using repetitive nonhydrolytic base cleavage of α-amino protective groups in Solid Phase Peptide Synthesis (SPPS). Protected [^{13}C] amino acid (AA) used in this SPPS model were prepared with 9-Fluorenylmethyloxycarbonyl (Fmoc) protecting group and with different acid labile side chain protecting group over AA type as: Fmoc-Ser(*tert*-Bu)-OH, Fmoc-Asp(*tert*-Bu)-OH, Fmoc-Arg(Pmc)-OH and Fmoc-Lys(Fmoc)-OH. All the protected AA were coupled by *N,N'*-dicyclohexyl carbodiimide (DCC) procedure, followed by Fmoc group cleavage using 20% piperidine in *N,N* dimethylacetamide (DMA). Quantitative deblocking side-chain AA protection and removal of KRDS from the solid support was effected by treatment with 50% trifluoroacetic acid in methylene chloride in a one pot-procedure. Homogeneous free [^{13}C] KRDS was obtained in 90% overall yield after HPLC purification. This synthesis schedule offered the advantage over present solid phase method which used acidolysis for repetitive α-amino group deblocking.

Key words: ester resin support; [^{13}C]Fmoc-amino acids; *tert*-Butyl and pentamethylchroman-sulphonyl protection group; solid phase synthesis; TFA cleavage

INTRODUCTION

KRDS is a particulary sequence of a milk peptide. Several investigations have shown the interest of this tetrapeptide as antifibronectine agents (1). It was necessary to label this peptide on each AA to study its biological activities and especially *in vivo*.

Althought the classical system of Merrifield (2), SPPS has been most used for synthesis of many large sequence peptides, other combinations of resin protecting groups, blocking groups, deprotection and cleavage reagents are possible as described by Steward (3) and Bodanszky (4). While the classical system can be expected to yield satisfactory results (5) for synthesis of most small peptides, for larger peptides and for special purposes, the choice of the proper combination of these factors can make the difference between success and failure. The strategy of the SPPS and the

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protected AA used are very important points in our Solid Phase Synthesis (SPS) scheme because of the final small yield of a ^{13}C-labelled tetrapeptide.

At the present time, there are two main strategies for synthesis of peptides by the Merrifield solid phase method, the first of which is essentially that due to Merrifield (6) where the repetitive N-protection/deprotection processes depend upon mild acid cleavage (trifluoroacetic acid - TFA) of the N-tert-Butyloxycarbonyl group (Boc). A consequence of this is that the side chain protection protocol and peptide-linker attachment to the resin rely upon more acid stable groups which require strong acids (HF or TFA) for final liberation of the peptide product. The other approach to SPPS utilizes the base-labile Fmoc group for N-protection which was designed by Carpino (7) and applied elegantly independently, by Meienhofer (8) and Atherton (9) to SPPS. This strategy involves the use of side chain protecting groups which may be cleaved by mild acid (10).

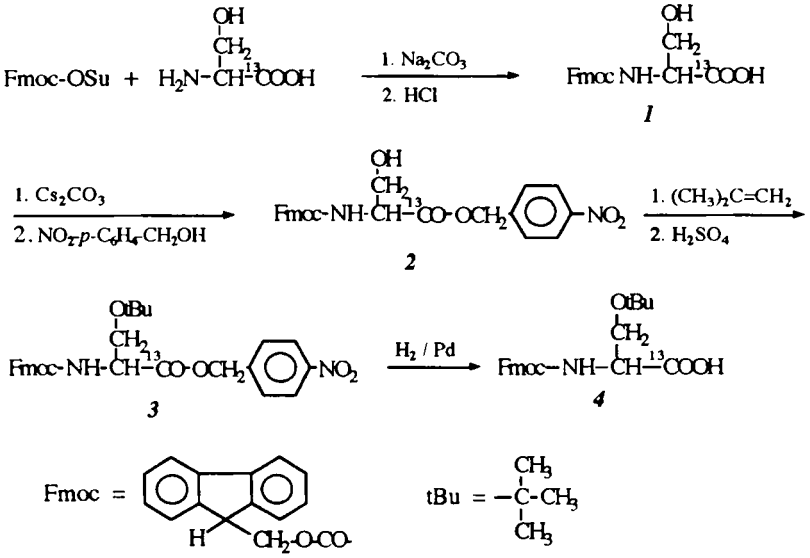
In the procedures discussed above, the selectivity of removal of the protecting group at each step of the synthesis and of the side-chain blocking groups (and cleavage of the peptide-resin link) at the end of the synthesis depends upon differences in rate acidolytic cleavage of theses two categories of groups. Problems attendant upon the use of such sytems have been already discussed (11-13). To overcome theses dificulties, orthogonal systems of SPPS have been developed (13, 14). In these systems the side-chain blocking groups and the peptide-resin link are completely stable to the reagent used to remove the α-protecting group at each step of the synthesis. As mentionned above, more attention recently has been given to the use of Fmoc-AA during SPPS with acid labile resin and excellent results were reported in syntheses of the model tetrapeptide Leu-Ala-Gly-Val (13), human β-endorphin (15), and dihydrosomatostatin (8). The main advantages in using N-Fmoc AA resides - in the elimination of repetitive acidolysis in each cycle, - in the rapid cleavage under mild conditions e.g. by piperidine in aprotic solvent,- in the ensuing opportunity of using tert-Butyl side-chain protection (16) and peptide to resin anchorage cleavable by mild acidolysis (17) thus avoiding the destructive final HF cleavage. Generally as peptide become more complex they become less stable toward harsh reagents such as HF or TFA. For these reasons, we have selected a labile peptide-resin link such as the p-alkoxybenzyl alcohol resin [polystyrene-1% divinylbenzene] of Wang (18) for the tetrapeptide [^{13}C] KRDS synthesis. This ether resin, while much more labile than the standard Merrifield resin to acidolysis, is much more stable to aminolysis. The peptide attached to this resin may be cleaved by TFA-dichloromethane (DCM) (50:50) (13). Furthermore, all the synthetized protected AA for use in the orthogonal system SPPS should be compatible with the same final deprotection step. So, we have synthetized Fmoc-AA with side-chain functional groups as tert-Butyl-derived blocking group (Fmoc-Ser(*tert*-Bu)-OH, Fmoc-Asp(*tert*-Bu)-OH), or arylsulphonyl derivated blocking group (Fmoc-Arg(Pmc)-OH) which have been reported previously as satisfactory to TFA cleavage (16,19,20).

This communication describes the preparation of [^{13}C]N$^{\alpha}$-Fmoc AA with side chain protecting group and experimental details of an exploratory study on repetitive base cleavage of Fmoc-AA and DCC-coupling in SPPS using the p-alkoxybenzyl ester peptide to resin anchorage.

RESULTS AND DISCUSSION

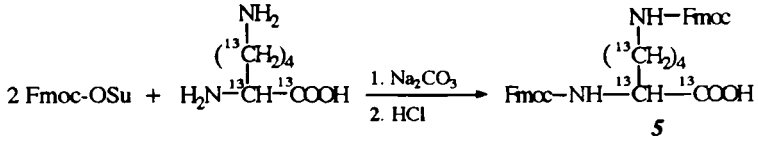
Synthesis of [^{13}C] N-Fmoc-Amino Acid side chain protected group

The [^{13}C]-Fmoc-Ser(*tert*-Bu)-OH **4** was synthetized in the similar manner as reported previously by Chang (16) and Wünsch (21). Generally, the Fmoc group was innitially introduced in the AA via Fmoc-chloride (Fmoc-Cl). However, it was found that the reaction of free AA with Fmoc-Cl leads to the formation of small amounts of Fmoc-dipeptides which are tedious contaminants and difficult to remove (22). Recently, a useful method has been presented to overcome those difficulties by using N-hydroxysuccinimide derivative (23). So, [^{13}C]Fmoc-Ser(*tert*-Bu)-OH was prepared by an alternative route (Scheme 1) via the intermediate N-Fmoc-AA and their benzyl esters and acid-catalyzed tert-butylation by isobutylene (16).



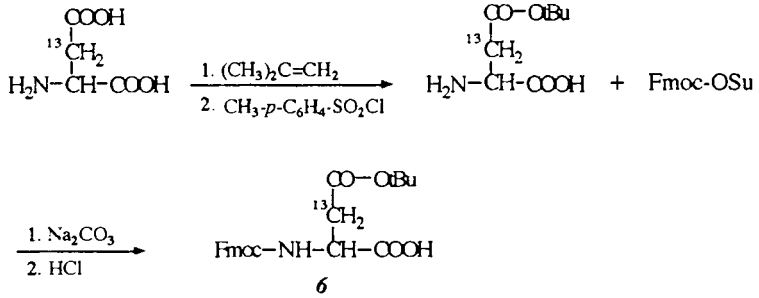
Scheme 1

[^{13}C]Fmoc-Lys(Fmoc)-OH (**5**) was synthetized from [^{13}C]-lysine by Fmoc N-protecting group procedure mentionned above using 9-fluorenylmethylsuccinimidyl carbonate (Fmoc-OSu) (22,24) to prevent dipeptide formation (see Scheme 2).



Scheme 2

For the preparation of [^{13}C]Fmoc-Asp(*tert*-Bu)-OH **6**, the standard manipulation used has already been well documented (19). Overall yield of **6** by previously reported multistep sequences (25, 26) were typically low (10-40% yield). However, the compound **6** synthesis from [^{13}C] aspartic acid by the usual method (26) was not satisfactory for the use of [^{13}C] compounds. So, the protected aspartic acid was prepared using a methodology recently describes by Lajoie (27) In fact, **6** was obtained from aspartic acid by treatment with isobutylene and 4-toluenesulfonic acid followed by Fmoc-succinimide in a novel two-step, one pot-procedure with moderate yield (see Scheme 3). This procedure has the advantage that the Fmoc N-protecting group was introduced without prior isolation or purification of the aspartic tert-butyl esters. Fmoc-OSu was added directly to the tert-butylation reaction mixture in the presence of aqueous carbonate to give the desired product **6** in 20-30%.yield The major byproduct of the reaction was the corresponding α-tert-butyl ester, which can be easily separated by column chromatography.



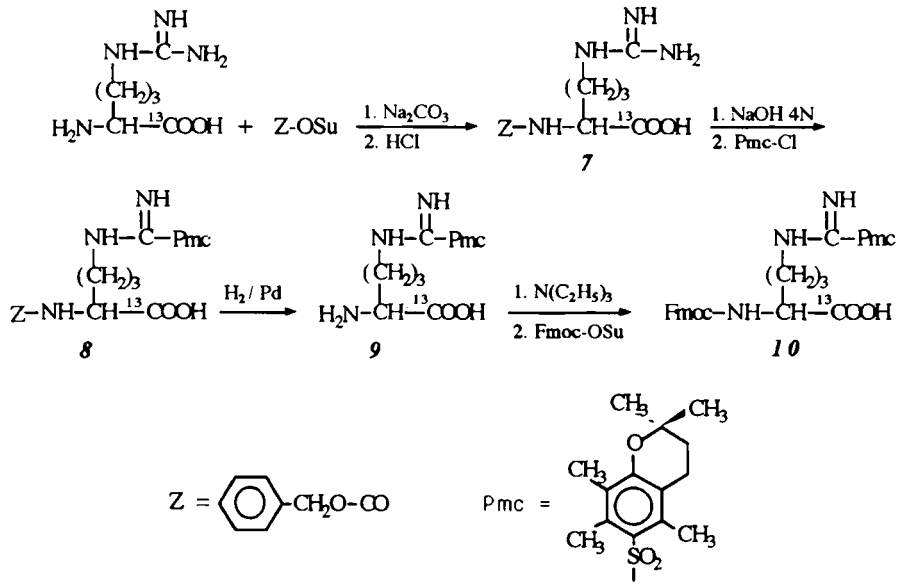
Scheme 3

Arginine still represents a problem, due to the difficulty of finding a protecting group of the guanidine side chain which would be very stable to bases and totally cleavable by mild acidolysis. For this reason, several protecting group have been used such as Boc (16), Adoc (28) and some multisubstituted benzenesulfonyl groups (29). In the choice of arylsulfonyl compounds available for this protection extended with the contribution of Fujino (30), Nishimura (31) and recently Ramage (32), the most acid labile of these derivative appears to be the N-2,2,5,7,8-pentamethylchroman-6-sulphonyl (Pmc) group. It can be cleaved by treatment with TFA/DCM (50:50) during 1 h (29). This group has been used, in association with the base labile Fmoc N-protecting group for the synthesis of arginine-containing peptides (20). So, we have synthetized [^{13}C]Fmoc-Arg(Pmc)-OH **10** from [1-^{13}C] arginine according to the procedure describes by Nishimura with the N-protecting group (31) and Fujino (19) via the Z N-protecting group (Scheme 4).

The physical properties of [^{13}C]Fmoc-Amino Acid side chain protected group were identical to those prepared by the standard procedure which were referenced in the literature.

Solid Phase Synthesis procedure

Manual SPPS must be carried out in a vessel which will contain the resin and provide a suitable means for addition of solvents and reagents, removal of them through a filter, and agitation of the resin with the liquids, all with protection from atmospheric moisture. We have used an apparatus specially designed for synthesis of labelled peptides. It is constructed in a size appropriate to



Scheme 4

accommodate the desired amount of resin according to 15-20 ml of solvent per gram of resin describes by many authors. Furthermore, our designed reactions vessels have several important features necessary for good SPPS reaction as indicated by Barany and Merrfield study (33). - It provide for rapid and thorough suspension of the resin so that all beads are in good contact with the solvent without being subject to any grinding action (Figure 1; 1); - provide for rapid and convenient addition of solvents in such a way that vessel walls are washed down completely (Figure 1; 2);- provide for rapid and complte solvent removal (Figure 1; 3);- allows different solvent volumes to be used at will with equally efficient resin contact and allows convenient removal of samples for monitoring during synthesis (Figure 1; 4). Mixing was obtained by fixing the reaction vessel on a shaker (type Vari-Mix; Thermolyne; USA). This shaker have allowed 45° alternatively rotation from horizontal position. A motor speed provide different variations of speed mixing. Following shaking operations, the shaker can be stopped with the vessel in the upright position (shown in Figure 1) and allow thus removing solvents through the fritted disk by vacuum into a wash receiver.

Many peptides adhere tightly to glass surfaces, especially basic and hydrophobic peptides as lysine, arginine etc. This problem can be prevented by silanization of the glass (3). So, all SPPS apparatus glassware has been silanized by treatment of the glass with dichlorodimethyl silane, followed by dry methanol.

The direct attachment of Fmoc-Ser(*tert*-Bu)-OH onto support has been accomplished by DCC mediated coupling in the presence of *p*-dimethylaminopyridine (DMAP) (13,16). This methodology remains the most widely used despites dipeptides formation with several AA as glycine (34) or partially racemization (35). As addition of DMAP has been reported to cause racemization side reaction, which can become a greater problem in prolonged, slow coupling reactions, we have used less than one equivalent of DMAP in DCC-Fmoc-Ser-resin coupling reaction. Different trials based on the excess of protected-AA, DMAP quantities and reaction times have allow to determine the better

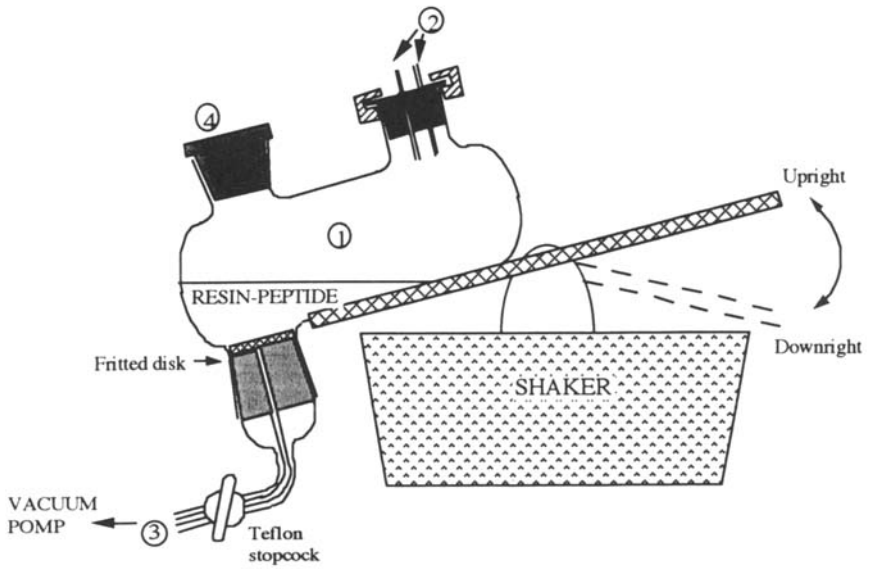


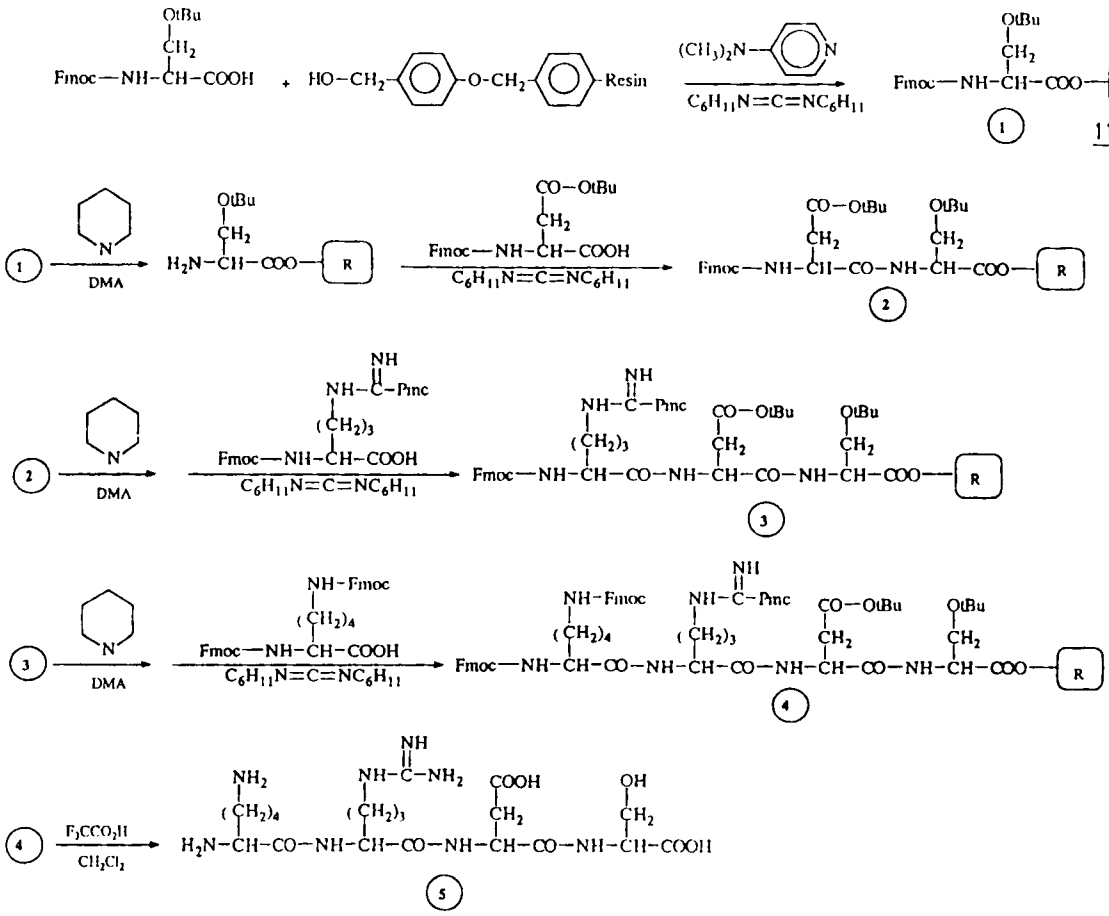
Figure 1: Apparatus used to tetrapeptide ^{13}C synthesis.

concentration of the reactants giving the highest substitution level of Fmoc-AA linked. If the degree of the AA-substitution is two low, it will not be possible to repeat the coupling reaction cause of the initial limited quantity of [^{13}C] first protected-AA (**4**). Attachment of Fmoc-Ser(*tert*-Bu)-OH to resin have provided a substitution level of 0.5 mmol per gram of resin by using two fold-excess (3, 14) of protected Serine, one equiv of DCC, and 0.1 equivalent of DMAP[1].

Coupling was effected in similar manner of Fmoc-Ser(*tert*-Bu)-resin reaction, with the three [^{13}C]Fmoc protected-AA (Scheme 5) in presence of DCC without DMAP because this compound has been reported to cause racemization in most case (36, 37). In DCC-mediated coupling reactions, the isourea intermediate rearranges spontaneously to the N-acyl urea. The acyl-urea is not reactive and represents undesirable loss of activated protected AA from the coupling reaction. The rate of this undesirable rearrangment is increased in N,N-dimethylformamide (DMF) and therefore the amount of DMF present in DCC-mediated coupling reactions is kept to the minimum. This problem has been highly diminished in the tetrapeptide synthesis by dissolution of the [^{13}C]Fmoc protected-AA in dichloromethane only (3). Since the rate of the desired coupling reaction is increased with increasing concentration, DCC-mediated coupling reactions are usually carried out in the minimum of solvent needed to dissolve the N-Fmoc-AA and swell the resin. Loss of activated intermediate by the rearrangement, as well as, the need to maintain a good rate of coupling until the last of the peptide is elongated, make the use of large excesses of Fmoc-AA and DCC mandatory in SPPS (3). The basic routine procedure recommended by many authors calls for the use of a 2,5-fold excess of N-Fmoc-AA and DCC over peptide amino group.

Table 1 shows the schedule adapted to KRDS synthesis. Detailed instructions are given by step number. The specification of the schedule have been chosen refering to the direction of Stewart and

[1] Greatest success in practical SPPS will be achieved if all operations are made standard and routine. All reactions will be scalled to the amount of resin.u



Scheme 5 : Total synthesis of KRDS tetrapeptide

Young (3) for the SPPS. The peptide-resin link is acid labile but it is stable under the piperidine treatment used to remove the Fmoc-group (38). All washes routinely effected with DMF have been replaced by DMA washes cause of the more stability of the Fmoc group in this solvent (38). When attaching the third [^{13}C] AA **5** in a peptide (working with a deprotected dipeptide-resin), the delay during these washes must be reduced. Loss of dipeptide by diketopiperazine formation has been reported (3). This cyclization can take place when the third residue was being added to the peptide. DCC and N-Fmoc protected-AA addition's in a reverse order will minimize this side reaction (3).

TABLE 1: Analytical data for synthetized [1-^{13}C] products

[1-^{13}C] Products	Yield (%)	m.p. (°C) Ref lit. [1-^{13}C]AA	t.l.c. $CHCl_3$-MeOH-HOAc	Rf	Formula (Mol wt)
1	72	87-88 / 88-90	90-6-4	0.33	$C_{18}H_{17}NO_5$ (328)
2	67	97-99 / 99-100	90-6-4	O.71	$C_{25}H_{23}N_2O_7$ (463)
3	60	69-71 / 70-72	98-2-1	0.65	$C_{29}H_{31}N_2O_7$(519)
4[a]	70	127-130 / 126-129	96-3-1	0.31	$C_{22}H_{25}NO_5$ (385)
5	20[b]	148-150 / 147-149	90-6-4	0.33	$C_{23}H_{25}NO_6$ (412)
6	53[c]	133-136 / 132-135	95-5-1	0.85	$C_{36}H_{34}N_2O_7$(597)
7	80	183-185 / 184-186	70-25-5	0.43	$C_{14}H_{20}N_4O_4$(309)
8	50	non identified	95-4-1	0.62	$C_{28}H_{38}N_4O_7S$(575)
9	75	184-186 / 182-186	94-6-1	0.20	$C_{20}H_{32}N_4O_5S$(442)
10[d]	66	95-110 / 105-111	95-5-1	0.58	$C_{35}H_{42}N_4O_7S$(663)

[a] Overall yield over 4 steps based on [1-^{13}C] H-Ser-OH: 27%; [b] Fmoc-AA obtained over 1 steps based on [U-^{13}C] H-Lys-OH, HCl; [c] Fmoc-AA obtained over 1 steps based on [3-^{13}C] H-Asp-OH; [d] Overall yield over 4 steps based on [1-^{13}C] H-Arg-OH : 22%

After 30 minute of coupling, the Kaiser (39) ninhydrin test[1] has been performed on a small amount of peptide resin to identify total coupling reaction. The sequential operation of the shedule for [^{13}C] KRDS synthesis should be performed until step 7 after the addition of the last protected-AA ([^{13}C] Fmoc-Lys(Fmoc)-OH). In this manner, the tetrapeptide-resin (Lys-Arg(Pmc)Asp(tBu)-Ser(*tert*-Bu)-Res) has been obtained with the lysine residue completely deprotected of the N^{α} and N^{ε} Fmoc protecting group cause it's an C-terminal residue. The final deprotection will be achieved in one step both to deblocking tert-Butyl and Pmc derived blocking group and to cleave linked peptide from the resin with 50% TFA in DCM (32). During deprotection step by mild acidolysis, the [^{13}C] tetrapeptide was exposed to a large concentration of tert-Butyl carbonium ions which are strongly alkylating agents. To prevent this side-reaction, an extremely effective scavenger used in TFA-DCM

[1] The Kaiser test: After prelevement of an aliquot of peptide resin, wash it with EtOH/AcOH (1:1) (2 times) and EtOH (2 times). Put few mg of the peptide resin in a test tube. Added 3 drops of reagents 1, 2, 3 successively and place the tube in a preheated block at 100°C for five minutes. Observe the color of the beads and the solution . The test is negative and the coupling is complete if the solution is yellow and the beads are white. The degree of coupling and the color of the beads and solution were determinated by Kaiser et al (38).

Reagents: 1- Cyanide: 2ml 0.01 M KCN diluted to 100 ml with pyridine; 2- Ninhydrin: 500 mg of ninhydrin in 100 ml n-BuOH; 3- Phenol: 80 mg of phenol in 20 ml of n-BuOH.

solution has proven efficacy and particularly with tryptophane (40). The tetrapeptide was obtained by 1 hour treatment with TFA in glassware as a crystalline brown mass upon evaporation.

After deprotection and several washes with TFA, the peptide was separated from the blocking groups and isolated by TFA extraction procedure (3). This methodology has the advantage that the peptide was obtained as a dry powder after repeated precipitation and centrifugation in ether.

Further filtration on Millipore filter and purification by High Performance Liquid Chromatography (HPLC) system provided analytically pure tetrapeptide [^{13}C] KRDS in 90% overall yield based on the starting Fmoc-Ser(*tert*-Bu)-resin.

EXPERIMENTAL PROCEDURES

Melting points were determined in a capillary tube with Büchi 510 apparatus and are uncorrected. [1-^{13}C] amino acids were purchased from CEA (Saclay, France). Silica gel 60 prepacked chromatograpy column were purchased from Merck. For thin layer chromatographie (t.l.c.), precoated silica gel G-60 (F_{254}) plastic plates were used from Merck with sample loads of 20-50 µg, solvent front 10 cm. All reference [^{12}C] AA protected as Z-Arg, Fmoc-Ser(*tert*-Bu)-OH, *p*-alkoxybenzyl alcohol resin (0.9 mequivalent/gram) etc were purchased from Novabiochem and Bachem (Switzerland). Infrared spectra were obtained on a Perkin Elmer 1310 grating spectrophotometer. D/Ci MS were carried out by the CERMAV (Centre d'Etude et de Recherche des Macromolécules Végétales, Joseph-Fourier University, Grenoble). Piperidine and dichloromethane were purchased from SDS and were spectrosol solvent. Dichloromethane was dried over calcium hydride. Toluene and methanol were distilled and dried over molecular sieves. Other solvents and chemicals were reagent grade and used without further purification (Carlo Erba). The shaker and the glassware illustrated by Figure 1 have been obtained respectively from Barnstead/Thermolyne, USA and Bioblock, France Nitrogen analysis were carried out from Microanalysis Center, Vernaison, France. Peptide sequence analysis was performed to Centre d'Etude Nucléaire de Grenoble (CENG), ^{13}C n.m.r. spectra were obtained respectively on 200 MHz and 400 MHz Bruker instruments using D_2O as solvent.

Synthesis of [^{13}C]N$^{\alpha}$-9-fluorenylmethyloxycarbonyl-O-tert-butyl-L-serine

[^{13}C]N$^{\alpha}$-9-fluorenylmethyloxycarbonyl-L-serine 1

[1-^{13}C]H-Ser-OH (300 mg, 2.8 mmol) was dissolved with stirring in 10% Na_2C0_3 (6 ml) for 0.5 h and cooled in an ice-bath. Dioxane (1 ml) was added followed by the slow addition of a solution of Fmoc-OSu (980 mg, 2.9 mmol) in dioxane (4.5 ml). The mixture was stirred for 1 h at 0°C and for 1.5 h at room temperature. After solvents evaporation, the reaction mixture was poured into ice-water (200 ml) and extracted with ether (3 x 150 ml). The aqueous layer was chilled in an ice-bath and acidified with conc. HCl to pH 2.0. The mixture was kept overnight at 0°C. Crystalline precipitates were collected by filtration, washed thoroughly with 0.1N HCl (2 x 100 ml) and H_2O (3 x 100 ml). After drying (Na_2S0_4) the precipitate was recrystallized from ethylacetate-petroleum ether (1:1) to yield 656 mg (2 mmol, 72 %) of t.l.c. homogeneous 1.Verification by UV and ninhydrin test. m.p. 88-90°.(see Table 1) D/Ci-MS (NH_3 + isobutane) (m/z) 385 (30), 328 (M^+< 2%), 106 (83).

[^{13}C]N$^{\alpha}$-9-fluorenylmethyloxycarbonyl-L-serine-O-p-nitrobenzyl 2

For conversion to the *p*-nitrobenzyl ester, a solution of [^{13}C] Fmoc-Ser-OH (2 mmol) in 80% EtOH (7 ml) was titrated with a 30% aqueous Cs_2CO_3 solution to pH 7.0. Solvent was removed by repeated evaporation in the presence of EtOH. The crystalline residue was suspended in dry DMF (7ml) and *p*-nitrobenzyl bromide (475 mg, 2.2 mmol) was added with stirring at 0°C The mixture was stirred at room temperature for one day. Precipitated salt was then filtered off and the filtrate concentrated in vacuo. The residual syrup was poured into ice-water and extracted with ether (3 x 10 ml) The combined extracts washed successively with aqueous saturated $NaHCO_3$, H_2O, 0.1N HCl, H_2O, dried and evaporated to a crystalline mass. It was purified by column gel chromatography with solvents system chloroform-methanol-acetic acid (90:6:4) to yield 625 mg (1.35 mmol, 67%) of yellow pure product **2**. (see Table 1), m.p. 97-98°C. I.R. (KBr) 1750 cm^{-1} (ester) and 3300 cm^{-1} (OH). D/Ci-MS (NH_3 + isobutane) (m / z) 464 (M^++ 1, 4%), 481 (M^++ NH_3, 35%), 520 (M^++ isobutane, 60%), 481 (50), 302 (75).

[^{13}C]N$^{\alpha}$-9-fluorenylmethyloxycarbonyl-O-tert.-butyl-L-serine-O-p-nitrobenzyl 3

P-nitrobenzyl ester **2** (1.35 mmol) was dissolved in dioxane-ether (2:1) solution (1.5 ml). The solution was placed in a vial sealed flask (reactivial) and cooled in an ice-water bath. The mixture was treated with excess isobutylene (350 mBar) (1,8 ml) and after rapid stirring, conc. H_2SO_4 (60 µl) was added with caution via a syringe. The flask was wrapped in a towel and kept at room temperature for one day. Excess isobutylene was evaporated after chilling and pressure release. The clear solution was poured into ice aqueous $NaHCO_3$ (10 ml) and extracted with ether. The organic extract was washed with H_2O, 0.1N HCl, H_2O, dried and evaporated to give a yellow crystalline residue. It was purified on column gel chromatography with chloroform-acetic acid (98:2) as eluant to yield 315 mg (0.6 mmol, 45%) of clear yellow crystalline needles **3** The product was analytically and chromatographically pure as compared with commercial standard reference (see Table 1). m.p. 70-71°C. I.R. (KBr): 1750 cm^{-1} (ester). D/Ci-MS (NH_3 + isobutane) (m / z) 519 (M^+, 20%), 391(25), 298 (75), 122 (100).

[^{13}C]N$^{\alpha}$-9-fluorenylmethyloxycarbonyl-O$^{\beta}$-tert.-butyl-L-serine 4

The fully blocked amino acid **3** (315 mg, 0.6 mmol) was dissolved in ethylacetate-ethyl alcohol (4:2 ml) and hydrogenated for 1 h at atmospheric pressure and room temperature with a gentle stream of H_2 in the presence of Pd black (24 mg). After removal of the catalyst, the filtrate was concentrated to a syrup. The viscous material was purified on silica gel column chromatography with solvent system $CHCl_3$-MeOH-HOAc (98:2:2) to yield 160 mg (0.42 mmol, 70%) of **4** which crystallized in fines needles. Compound **4** was analytically and chromatographically pure as compared with commercial standard reference, m.p. 127-129°C (see Table1). I.R. (KBr) 3420 (NH) and 1755 cm^{-1} (COOH). D/Ci-MS (NH_3 + isobutane) (m / z) 384 (M^+, < 1%) , 328 (10), 195 (88), 162 (35).

Overall yield over 4 steps based on [1-^{13}C] H-Ser-OH: 27%

Synthesis of [^{13}C] N$^{\alpha}$-9-fluorenylmethyloxycarbonyl-O-tert.-butyl-L-aspartic acid

[^{13}C]N$^{\alpha}$-9-fluorenylmethyloxycarbonyl-O-tert.-butyl-L-aspartic acid 5

A mixture of [3-^{13}C] H-Asp-OH (300 mg, 2.2 mmol) and *p*-toluenesulfonic acid (TsOH) (2 eq., 857 mg) in dioxane (11 ml) was stirred under isobutene gas (350 mBar) (10 ml) with a magnetic stirring rod for 3 days at room temperature. Following the addition of aqueous 10% Na_2CO_3 (14 ml),

Fmoc-OSu (2.2 mmol, 760 mg) in dioxane (4 ml) was added dropwise at O°C. After stirring overnight at room temperature, the mixture was poured into ice water (100 ml) and washed with ether (4 x 25 ml) to remove excess Fmoc-OSu and Fmoc-di-tert-butyl-Asp-OH. The aqueous phase was chilled (0°C), acidified to pH 5.5 with 1N HCl, and extracted with EtOAc (3 x 150 ml). The combinated organic fractions were washed with saturated NaCl solution, H_2O and dried (Na_2S0_4). The solvent was removed in vacuo yielding a white solid mixture of mono α and β -tert-butyl esters. The product was dissolved in CH_2Cl_2-petroleum ether (1:1) at 30°-60°C and chilled overnight at 0°C. The resulting crystals were filtered and washed with petroleum ether (30°-60°C), giving the mono β-tert-butyl ester **5** as the only product (> 95% pure/ mono α). The ester **5** was chromatographed through a silica gel G-60 open column, eluted with $CHCl_3$-MeOH-HOAc (90:5:5) to remove most impurities. Subsequently, the derivative crystallized. Yield: 20%, 0.44 mmol, 181 mg. M.p.: 147-149°C. I.R. (KBr) 1755 cm^{-1} (COOH). Compound **5** was identified by t.l.c as compared with commercial standard. D/Ci MS (m/z) 412 (M^+, 2%), 356 (15),179 (100), 134 (21).

Synthesis of [^{13}C] N$^{\alpha}$-9-fluorenylmethyloxycarbonyl-N$^{\varepsilon}$-9-fluorenylmethyloxy carbonyl-L-lysine

***[^{13}C]N$^{\alpha}$-9-fluorenylmethyloxycarbonyl-N$^{\varepsilon}$-9-fluorenylmethyloxycarbonyl-L-lysine* 6**

[U-^{13}C]H-Lys-OH, HCl, H_2O (168 mg, 0.87 mmol) was dissolved with stirring in 10% Na_2C0_3 (4 ml) for 1 h and cooled in an ice-bath. Dioxane (1 ml) was added followed by the slow addition of a solution of Fmoc-OSu (2 eq., 591 mg, 1.75 mmol) in dioxane (3 ml). The mixture was stirred for 0.5 h at 0°C and for 3 h at room temperature. The reaction mixture was poured into ice-water (200 ml) and extracted with ether (3 x 150 ml). The aqueous layer was chilled in an ice-bath and acidified with conc. HCl to pH 2.0. The mixture vas allowed overnight at 0°C. Precipitates were collected by filtration, washed thoroughly with 0.1N HCl (2 x 100 ml) and H_2O (3 x 100 ml). After drying (Na_2S0_4) the precipitate was partially dissolved in acetone and filtered. The filtrate was evaporated in vacuo giving crystalline white product **6**. It was chromatographed through a silica gel G-60 open column, eluted with $CHCl_3$-MeOH-HOAc (96:4:1) to remove most impurities. Yield 57% (299 mg, 0.5 mmol). Homogeneous t.l.c. of **6** as compared with a commercial standard. Verification by UV and ninhydrin test negatifve. M.p. 132-135°C (see Table 1). D/Ci MS (m/z) 599 (M^+, <1%), 377 (95), 155 (32).

Synthesis of [^{13}C] N$^{\alpha}$-9-fluorenylmethyloxycarbonyl-N^{G}-2,2,5,7,8-pentamethyl chroman-6-sulphonyl-L-arginine 10

***[^{13}C]N$^{\alpha}$-benzyloxycarbonyl-L-arginine* 7**

[1-^{13}C]H-Arg-OH (300 mg, 1.71 mmol) was dissolved in ice cold N NaOH (0.55 ml) and 0.2 ml of dioxane with stirring. Both stirring and cooling to about 0°C are continued while benzyloxy succinimidyl carbonyl (Z-OSu) (1.3 eq., 555 mg, 2.2 mmol) in dioxane (1.4 ml) and 2N NaOH are added alternately, in a few portions. The pH of the mixture was kept between 9 and 10. After addition of the reactants, stirring of the suspension was continued until the pH drops to 7-7.5. The precipitate was collected on a filter, washed with cold water and recrystallized from boiling water. Crystallization was completed in the cold. The result product was collected, dried and suspended in acetone, filtered and washed with acetone and ether. Purified **7** was obtained in a finely powdered

form with yield of 80% (417 mg, 1.34 mmol). M.p.: 184-187°C. D/Ci MS (m/z) 308 (M^+ < 2%), 267 (100), 172 (2), 134 (60).

[^{13}C]N$^{\alpha}$-benzyloxycarbonyl-N^{G}-2,2,5,7,8-pentamethylchroman-6-sulphonyl-L-arginine 8

A suspension of **7** (417 mg, 1.35 mmol) in a mixture of distilled water (1.5 ml) and acetone (6 ml) was cooled in ice-water bath. Precooled 4N NaOH (1.5 ml) was added with vigourous stirring to bring the pH to 12 and to maintain it in this range. After complete dissolution of the starting material (1 h), a solution of Pmc-Cl (2,5 eq., 1.02 g, 3.4 mmol) in acetone (2.5 ml) was added dropwise. During the addition , pH must be maintained to 12 by addition of 4N NaOH to the cold reaction mixture. Stirring was continued in the cold for about 1 h. The mixture was neutralized with 10% citric acid solution and the solvent was evaporated off. The mixture was diluted with H_2O and extracted with Et_2O (3 x 100 ml). The aqueous phase was acidified to pH 3 with 6N HCl and extracted with AcOEt (4 x 150 ml). Organic phase was washed with saturated NaCl solution, dried over Na_2SO_4 and concentrated. The residue was purified by column chromatography on silica gel with solvent system $CHCl_3$-MeOH-HOAc (95:4:1) to yield 385 mg (0.67 mmol, 50%) of **8** as a white solid. Homogeneous t.l.c.of [U-^{13}C] Z-Arg(Pmc)-OH was obtained as compared with commercial standard. Verification by UV and ninhydrin test negatif (see Table 1). D/Ci MS (m/z) 631 (+isobutane) 575 (M^+, <1%), 440 (2), 307 (29), 205 (100).

[^{13}C]N^{G}-2,2,5,7,8-pentamethylchroman-6-sulphonyl-L-arginine 9

A methanolic solution (9 ml) of **8** (385 mg, 0.67 mmol) was hydrogenated over Pd black (36 mg) as catalyst . The mixture was stirred at room temperature. After 2 h, the catalyst was filtered off and the solution evaporated in vacuo. The material was triturated with Et_2O to give a precipitate. The solid was filtered and washed with Et_2O. The dry crystalline derivative **9** was obtained by recrystallization in MeOH-Et_2O (1:1). Yield: 220 mg (0.5 mmol, 75%), m.p.:.182-186°C. Identification by t.l.c. UV and ninhydrin test showed identical Rf as compared with commercial standard (see Table 1). D/Ci MS (m/z) 442 (M^+, <1%), 175 (85), 134 (28).

[^{13}C]N$^{\alpha}$-9-fluorenylmethyloxycarbonyl-N^{G}-2,2,5,7,8-pentamethylchroman-6-sulphonyl-L-arginine 10

A methanolic solution (3 ml) of **9** (0.5 mmol, 220 mg) was stirred in the presence of triethylamine (1.1 eq. 0.55 mmol, 77 µl). A solution of Fmoc-OSu (0.5 mmol, 168 mg) in dioxane (0.7 ml) was added dropwise. The solution was stirred for 1 h at room temperature and evaporated under reduced pressure to give an oil which was dissolved in EtOAc (75 ml). The organic solution was washed with 0.1 M citric acid (3 x 50 ml) and H_2O (3 x 50 ml), dried (Na_2SO_4) and concentrated in vacuo. The residue material was purified by column chromatography on silica gel with solvent system: $CHCl_3$-MeOH-HOAc (95:5:1) to yield 251 mg (0.38 mmol, 76%) of pure **10**. Homogeneous t.l.c.of **10** was obtained as compared with commercial standard (identical Rf). Verification by UV and ninhydrin test negative (see Table 1). M.p.:105-111°C. D/Ci MS (m/z) 663 (M^+, <1%), 441 (35), 395 (95), 173 (12).

Overall yield over 4 steps based on [1-^{13}C] H-Arg-OH: 22%

Silanization of glassware

The dry glassware was treated for 15 mn with dichlorodimethyl silane, 10% (V/V) in dry toluene. Be sure the entire surface is wetted with the reagent and that no air bubbles cling to it. Glassware was then rinsed 3 times with dry toluene. Do not dry. Immediately treat it with dry MeOH for 15 minutes then it must be rinsed well with MeOH and acetone, and dry thoroughly.

[^{13}C]N$^{\alpha}$-9-fluorenylmethyloxycarbonyl-O-tert.-butyl-L-serine p-alkoxybenzyl ester resin 11

p-Alkoxybenzyl alcohol resin (161 mg, 0.99 mmol/g resin) was put in the glassware and washed several times with and suspended in DCM (6 ml). Fmoc-Ser(*tert*-Bu)-OH (158 mg, 0.41 mmol, 2,5 eq./OH free on resin) was added and mixed 30 secondes. DMAP (0.1 eq./AA used) in first then DCC (1 eq./AA used) were added at 20°C and the ensuing suspension shaken for 1 hour. After removal of the solvent by vacuum aspiration, the mixture was washed successively with DMA (2 times), EtOH (2 times) and DCM (3 times). The resin was dried thoroughly in vacuo over P_2O_5. The Fmoc-Ser(*tert*-Bu)-OH content per gram of resin was determined to be ca. 0.5 mmol by nitrogen analysis (0.7% = 0.5 mmol/g) and yield 168 mg (0.084 mmol) of [^{13}C] protected serin linked to resin.

As we required 90 mg of KRDS, we used 0.2 mmol of protected serine-resin based on the overall yield of 85% obtained in SPPS synthesis of [^{12}C] KRDS with commercial protected AA. So, 168 mg of [^{13}C] Fmoc-Ser(*tert*-Bu)-resin (0.084 mmol) was added to 191 mg of [^{12}C]Fmoc-Ser(*tert*-Bu)-resin (0.115 mmol, 0.6 mmol/g resin) to give 360 mg (0.2 mmol) of ^{13}C/^{12}C **11** (42% of labelled [^{13}C] protected serine fixed to resin).

General solid-phase synthetic procedure (Table 2)

[^{13}C]L-Lysyl-N^{G}-2,2,5,7,8-pentamethylchroman-6-sulphonyl-L-arginyl-O-tert.-butyl-L-aspartyl-O-tert.-butyl-L-serine p-alkoxybenzyl ester resin

Fmoc-Ser(*tert*-Bu)-O-resin (360 mg, 0.2 mmol) was placed in the manual reactor illustrated by Figure 1. The protocol of an operational cycle is shown in Table 2 with steps 1 to 9. Resin was swollen with 6 ml of DCM, treated with piperidine-DMA (20% v/v), washed with DMA, dioxane/H_20 (1:1), DMA, DCM. Stepwise SPPS proceeded using single DCC[1]-mediated coupling (2,5 eq. = 0.5 mmol of the protected AA derivative ([^{13}C] Fmoc-Asp(*tert*-Bu)-OH)[2] and of DCC in a total of 5 ml DCM as solvent for 30 min, at which time ninhydrin tests were negative. There followed washings with DCM and the cycle was repeat with the two others ^{13}C protected-AA (third AA: [^{13}C]Fmoc-Asp(Pmc)-OH[3] and fourth AA : [^{13}C] Fmoc-Lys(Fmoc)-OH[4]).

For the addition of the third residue (Fmoc-Arg(Pmc)-OH), reverse the order of the step 8[5] The last cycle after the addition of the fourth AA was continued until the step 7 to deblocking the two

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[1] 1 M solution of DCC in DCM (0.206 g DCC/ml).

[2] [^{13}C] Fmoc-Asp(tBu)-OH) (0.38 mmol) was mixed with 0.12 mmol of [^{12}C] commercial analog to obtain 2.5 eq (0.5 mmol) of protected aspartic acid necessary to DCC coupling. (^{13}C labelled: 76%).

[3] [^{13}C]Fmoc-Asp(Pmc)-OH (0.44 mmol) was mixed with 0.05 mmol of [^{12}C] commercial analog to obtain 2.5 eq. (0.5 mmol) of protected arginine necessary to DCC coupling. (^{13}C labelled: 86%).

[4] To DCC coupling only [^{13}C]Fmoc-Lys(Fmoc)-OH have been used (^{13}C labelled: 100%). This derivate was sparingly soluble in DCM. So, it was dissolved in a mixture of 20% DMA in DCM. In this case, DMA should be added as a wash solvent after the coupling step 8.

[5] Add DCC before adding the Fmoc-AA. Use only a very brief mix after addition of DCC.

Fmoc group of the lysine. The L-Lysyl-L-Arginyl(Pmc)-L-Aspartyl(tBu)-L-Seryl(tBu)-O-resin was dried over P_2O_5 and stocked at 0°.C

TABLE 2: Manual schedule for the Fmoc-Based SPPS

Step	Manipulations	Reagent	Vol (ml)	Time (min)
1	Washing	DCM (2 times)	6-7	3
2	Washing	DMA[a] (2 times)	6-7	3
3	Deprotection[a]	20% piperidine/DMA	6-7	13
4	Washing	DMA (2 times)	6-7	3
5	Washing	Dioxane/H_2O (2:1) (2 times)	6-7	10
6	Washing	DMA (3 times)	6-7	5
7	Washing	DCM (3 times)	6-7	5
8	Coupling[b]	1- Fmoc-AA N^{ε}-protected (2.5 eq.) in DCM	5	1,5
		2- DCC (2.5 eq.)	0.45	30
9	Washing	DCM (4 times)	6-7	5

[a] Monitoring coupling by Kaiser test : negative for all AA coupling; yellow (Ser-Asp), brown (Ser-Asp-Arg), yellow-brown (Ser-Asp-Arg-Lys) ; [b] Monitoring deprotection by Kaiser test : positive: dark blue.

Cleavage experiment

L-[U-^{13}C]Lysyl-L-[1-^{13}C]Arginyl-L-[3-^{13}C]Aspartyl-L-[1-^{13}C]Serine (KRDS)

The L-Lysyl-L-Arginyl(Pmc)-L-Aspartyl(tBu)-L-Seryl(tBu)-O-resin was placed in the reactor and washed with DCM. Subsequently, it was treated with 50% TFA in DCM[1] (8 ml) for 1 h at 20°.

The resin was filtred off and washed 2 times with TFA/DCM[2]. After collecting the TFA extract, it has be evaporated in vacuum under N_2. The extract was then homogenized with TFA to obtain a TFA volume of about 5 mg per g of resin. Precipitation of the tetrapeptide was obtained by addition to the TFA solution of 5 volumes of cold Et_2O. After 30 min, the peptide was centifuged for 5 min at 2000 rpm in a centrifuge. The precipitate was repeatedly washed with cold Et_2O and centrifuge 4 times. Removal of the TFA and Et_2O was performed in desiccator coupled with a vacuum pump using a dry ice-EtOH trap. The tetrapeptide was obtained as a white powder (120 mg) which give homogeneous t.l.c. in n-BuOH:HOAc:H_2O (4:2:2). Rf 0.25.

The crude tetrapeptide thus obtained was purified by HPLC. A sample (40 mg) was applied to a Nucleosil C18 column (300 x 4.6, porosity 0.1µ, diameter 5 µ), which was eluted with solvent system: water-acetonitrile-TFA (970:30:1) at the flow rate of 1ml/min. The eluate corresponding to the main peak (retention time 4,5 min, detected by UV absorption measurement at 215 nm) was collected and the solvent evaporated in vavuo. The rest of the sample was similarly purified. The

[1] A scavenger such as indole at a concentration of 1mg/ml was added to TFA/DCM solution and must be allowed to stand overnight before using. The reagent should have a deep burgundy color.

[2] TFA must be let in contact with resin for about 1 min.

tetrapeptide was then dissolved in 4 ml of water and solvent was removed by lyophilization to give a fluffy white powder. Yield 90 mg. (176 μmol, 90% overall from [^{13}C] N^{α}-9-fluorenylmethyloxy carbonyl-O-tert.-butyl-L-serine p-alkoxybenzyl ester resin). T.l.c. in n-BuOH-HOAc-H_2O (4:3:3) showed a single red ninhidrin-positive spot at Rf = 0.38.

Peptide analysis to CENG have indicated the good sequence of AA almost free of impurities.

^{13}C n.m.r. (Carbon identity, ppm) quaternary carbons (1,171; 4 et 8, 169; 13, 158; 14, 172); tertiary carbons (3, 56; 5, 50; 9, 53; 15, 54); secondary carbon (2, 60.5; 6, 35; 10, 28; 11, 24; 12, 40; 16, 20.5; 17, 26; 18, 30; 19, 38.5). see scheme 6 to carbon identity.

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