Journal of Labelled Compounds and Radiopharmaceuticals—Vol. XXXI, No. 10

SYNTHESIS OF TRITIATED TRIPEPTIDES AND THEIR VERY SIMPLE PURIFICATION PROCEDURES

G. Angelini, C. Sparapani, A. Margonelli*

Istituto di Chimica Nucleare del C.N.R., C.P. 10, 00016 Monterotondo Stazione (Rome) Italy

* Polifarma S.p.A., Centro Ricerche, Via Tor Sapienza, 138, 00155 Rome Italy

SUMMARY

The complete reaction pathway (five steps) for the syntheses of tritiated [CBZ-Pro-*Leu-TrpOH] 7 (POL-443) and [Pyr-*Leu-TrpOMe] 5 (POL-509) starting from tritiated *Leu-[3, 4, 5-^{3}H(N)] is described. The products 5 and 7 are obtained with high level of chemical and radiochemical purity and in good total yield (70% for 5 and 60% for 7)(1). The use of the "Solid Phase Extraction" procedure made the purification and recovery steps very simple and effective with no isotopic exchange.

Key Words: Tritium, Peptide synthesis, Tritiated peptides, S.P.E. techniques, S.P.E. purification.

0362-4803/92/100739-19$14.50

Received 12 March, 1992
Revised 23 June, 1992

INTRODUCTION

Venoms extracted from crotalid and viperid snakes contain substantial amounts of small peptides, possibly related to important pharmacological activities (2). Some of the peptides, referred to as "Bradykinin Potentiating", have been very well studied in the past, and opened the way to the development of a new class of drugs, the ACE-inibitors, consisting of modified di- or tripeptides, used in hypertension (3).

The presence in the Crotalus Atrox snake venom of a decapeptide, called POL-236 (4) characterized by the original N-terminal tripeptide PYR-LEU-TRP, suggested that the structure might have pharmacological effects. Two tripeptides compounds, therefore, POL-443 and POL-509, were synthesized and subjected to animal experiments, in order to ascertain antihypertensive (5a) and immunological properties (5b).

With the aim to well understand the binding properties of these tripeptides with their physiological targets and their relative metabolic mechanisms, the use of the radioactive forms of the ligands (tripeptides) represents a valid strategy to pursue this goal (6-8).

Tritium is a well suitable isotope since its presence may not modify the properties of biological molecules (9) and its physical-chemical characteristics allows one to obtain a precise labelling in a well established part of the molecule (10-13).

Different synthetic procedures can be used for the preparation of tritium labeled peptides (14-18), summarized as follows: a) catalytic reduction (with molecular tritium) of suitable amino acids containing double (es: 3,4-dehydroproline and 4,5-dehydroleucine) (14-15) or triple bonds (es: 2,6 diamino-4-hexynoic acid) (16); b) reductive halogenolysis (with molecular tritium) of halogenated amino acids (14, 17-19).

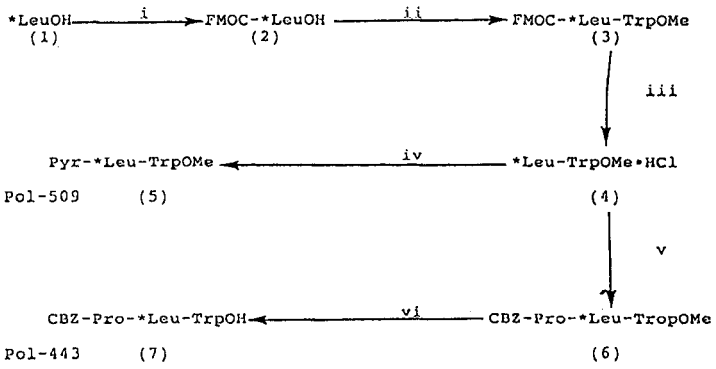
Both these methods require the insertion (sometimes very difficult) of the suitable amino acid precursor into the peptide chain before the reductive action of the tritium can take place. However isotopic exchange can not be excluded in particular when aromatic rings are present (18, 19).

A more recent approach (20) employs the enzymatic synthesis of the peptidic chain, starting with a previously labeled amino acid. Moreover the final tritiated products, owing to the presence of several collateral compounds (14) can be significantly impure and extensive and laborious purification procedures, involving often low reaction yields, may be necessary. Considering that the two tripeptides POL-509 and POL-443 show a common end dipeptidic structure, Leu-TrpOR, condensed to two very different amino acids ((L)-pyroglutamic acid and N-CBZ-(L)-proline respectively), each one of the described methods should have involved serious labelling problems. So a different method has been followed in this work (21). In fact we built the two tripeptides around to the central amino acid (leucine) previously labelled with tritium in suitable positions (Scheme 1).

In a such complex radio-synthetic scheme (see Scheme 1), only a careful choice, among the very large peptide-synthesis literature (22), of the synthetic procedure can avoid side-reactions and co-products that can involve extensive and laborious chemical and radiochemical purification procedures, so that the final products yields would result very low. These difficulties, which have to be avoided in every case, become unaceptable when radioactive compounds are involved.

As reported in this paper it is worth noting the convenient use of the aminic protective group (FMOC) (23) and the condensing agent (HBTU) (24) attaining the goal to have a synthetic process simple, clean and fast. In these conditions the eventual collateral products, formed during each steps of the Scheme 1, are

SCHEME I



i - $CH_3CN/H_2O/Na_2CO_3$/FMOC-OSu/r.t./3.5 hr.

ii - CH_3CN/HBTU/TEA/TrpOMe•HCl/r.t./3.5 hr.

iii - CH_3CN/DEA/r.t./30 min.

iv - CH_3CN/HBTU/TEA/PyrOH/r.t./2.5 hr.

v - CH_3CN/HBTU/TEA/CBZ-ProOH/r.t./3 hr.

vi - CH_3CN/H_2O/NaOH 0.1N/r.t./4 hr.

present in minimum amounts or absent at all, so that it was possible to use a general very simple purification method: the "Reverse-phase Solid Phase Extraction" (25). Fast and complete isolation of the products have been obtained during each step of the synthesis, notwithstanding the massive amount of the involved materials (about 100 mg). Moreover this purification technique, for the first time at our knowledge, has been applied to tritium labelled compounds, resulting very effective and reducing the isotope exchange at very low level or absent at all.

EXPERIMENTAL

Material: Pure tritiated (L)-Leucine-[3, 4, 5-^{3}H(N)] 1 (*LeuOH) was purchased from Sigma Chemical Co. and was used without further purification. It provided also (L)-Tryptophan methyl ester

hydrochloride, (L)-Pyroglutamic acid and (L)-CBZ-Proline acid (1). N-(9-Fluorenylmethoxycarbonyloxy)-succimide (FMOC-OSu), O-Benzotriazol-1-yl-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU) and N-(9-Fluorenylmethoxycarbony)-(L)-leucine (FMOC-LeuOH) were obtained from Fluka. Aldrich Chemical Co. provided diethyl and triethylamine (DEA and TEA). Methanol (MeOH), purified water, trifluoroacetic acid (TFA), acetonitrile (CH_3CN) and the solid phase extraction (SPE - C18 - 6 ml - HC) columns were purchased from Baker Co.

Methods: High performance liquid chromatography (HPLC) analyses were performed with Perkin Elmer Series 10 liquid chromatograph connected to a Dynamax C18 (300 A, 12 μ, 250 x 4.6 mm) (Rainin Co.) column on line with a Gilson U.V. mod. 112 detector. The analyses (total flow: 0.7 ml/m) were carried out using either an isocratic elution (condition A: 0.1% TFA mixture (70:30) of MeOH/H_2O) or a gradient elution (condition B: from a 0.1% TFA mixture (40:60) of MeOH/H_2O increased (20 min) at (70:30) and holded for 20 min). The comparison of the retention volumes with those of autenthic commercial standards or of previously prepared compounds by the Polifarma Research Center, allowed the identification of the reaction products and the check of the absence of impurities or eventual co-products whose formation could be possible.

Radiochemical analyses were determined, by connecting the HPLC instrument, "on line" to a liquid scintillation monitor (Berthold mod. LB 504) and using a "mixture cell" of 600 μl. Instagel (Packard) was used as scintillation cocktail (flow 1.4 ml/m). Static determination of the radioactivity during each step of the synthesis were obtained by liquid scintillation counting on the Packard Tri-Carb 2260 XL.

The purification of the crude products in each single step of the Scheme 1 was performed in a very fast and effective way, employing the SPE technique (25) with some modifications (vedi infra) at the usual procedure (25a). For every synthetic step, the SPE technique has been applied two or three times (depending on the products) as subsequent application of single procedures.

SPE-procedure. Each SPE-column has been previously washed with CH_3CN (3 ml). An accurate vacuum (380 torr) was applied so that the elutions of the washing CH_3CN and the subsequent mixture of solvents were favored allowing however the best interactions to warrant the products purification.

The SPE-column, completely eluted by CH_3CN, was slowly activated (1 ml/m) with the suitable solvent mixture leaving the SPE-column bed just covered by the solvent itself. The crude synthetic product in the same mixture of solvents was added and using the same flow (1 ml/m) and vacuum was extracted into the SPE-column. The eluting liquid was completely recovered, representing the mobile phase for the subsequent SPE procedure (if necessary). At this point, depending on the separative conditions, the purified product could be contained in the eluted liquid phase and its recovery obtained by simple evaporation or lyophilization or instead be absorbed into the SPE-column resin. In this case, it was recovered eluting the column with a suitable amount of CH_3CN.

Preparation of N-(9-Fluorenylmethoxycarbonyl)-leucine-[3,4,5-^{3}H(N)] [FMOC-*LeuOH] 2.

A solution of 1.11 Gbeq of *LeuOH-[3,4,5-^{3}H(N)] 1 (nominal specific activity: 5.77 TBeq/mmol) in about 30 ml of 2% aqueous ethanol was obtained collecting six purchased vials (same preparation lot). This collection was performed washing every vial, where the radioactive compound was contained, with 0.22 ml of HCl

0,2 N (0.264 meq). The resulting acid solution was diluted with 30 mg (0.229 mmol) of pure cold Leu and lyophilizated, providing about 38 mg (0.227 mmol) of *LeuOH•HCl (total activity: 1.07 GBeq and specific activity: 4.71 GBeq/mmol), as white and bulky solid. It was dissolved in 2.1 ml of H_2O/CH_3CN (1:1) mixture, cooled to 0°C and under stirring 72.8 mg (0.687 mmol) of Na_2CO_3 were added, giving a solution slightly basic. Then 75.6 mg (0.225 mmol) of FMOC-OSu and 1 ml of CH_3CN were further added. The mixture was stirred for 3.5 hours at r.t. and diluted with 3 ml of CH_3CN, 7 ml of H_2O and 0.7 ml of HCl 1N. The slightly opalescent liquid so obtained was passed through three SPE-columns which trapped the FMOC-*LeuOH 2 and permitted the elution of the co-products and impurities (HPLC-control). The FMOC-*LeuOH 2 was recovered from the SPE-columns with CH_3CN, that was dried for one night over anhydrous Na_2SO_4 and filtered off on SPE-column. A solution of 66 mg (0.187 mmol, yield 83.1%) of 2 in about 8 ml of CH_3CN was obtained. Radio-HPLC analysis (condition A) allowed to verify the high chemical and radiochemical purity (>99%) of 2. Total activity: 880 Mbeq and specific activity: 4.70 GBeq/mmol.

Preparation of N-(9-Fluorenylmethoxycarbonyl)-leucine-[3,4,5-^{3}H(N)]-tryptophan-methylester [FMOC-*Leu-TrpOMe] 3.

The solution containing 2 was evaporated at r.t. under vacuum at r.t. up to 4 ml. At 0°C and under stirring an excess of TrpOMe•HCl (63 mg, 0.247 mmol) followed by 70 µl (0.5 mmol) of TEA, 94 mg (0.239 mmol) of HBTU and 1 ml of CH_3CN were added. The mixture was stirred in the dark at r.t. for 3.5 hours and diluted with 5 ml of CH_3CN, 8 ml of H_2O and 2 ml of HCl 1N. A slight opalescence was noted. The mixture was passed through three SPE-columns, which trapped the FMOC-*Leu-TrpOMe 3. Their elution with CH_3CN permitted the purification of 3 and its radioactive solution was dried for one night over anhydrous Na_2SO_4 and then

filtered off on SPE-column. Radio-HPLC analysis (condition A) showed the presence of 3 (97 mg, 0.175 mmol, 93%) at high level of chemical (97%) and radiochemical (99%) purity. Total activity: 821 MBeq and specific activity: 4.70 GBeq/mmol.

Preparation of Leucine-[3,4,5-^{3}H(N)]-tryptophan-methylester hydrochloride [*Leu-TrpOMe•HCl] 4.

The solution containing 3 was concentrated at r.t under vacuum, up to 5,5 ml. At 0°C a large excess of DEA (1.1 ml, 10.6 mmol) was added to the stirred solution. After 30 minutes the solution was rapidly transferred into the rotavapor and almost completely evaporated at r.t.. This operation was repeated after the addition of other 5 ml of CH_3CN. The oily brown product so obtained was diluted with 9 ml of CH_3CN, 3 ml of HCl 0.2 N and 9 ml of H_2O. The mixture was stirred for 15 minutes producing a milky emulsion. In order to purify the product 4 from the residue starting compound 3, from the dibenzofulvene (expected as co-product) and eventually from the undesired radioactive diketopiperazine, the emulsion was passed through three SPE-columns. This procedure efficiently trapped the co-products and the starting compound, providing an aqueous acid solution containing 4 (63 mg, 0.171 mmol, 98%). Radio-HPLC analysis (condition B) showed the high level (>98%) of chemical and radiochemical purity of 4. Total activity: 799 MBeq and specific activity: 4.67 GBeq/mmol. Radio-HPLC analysis of the compounds trapped in the SPE-columns confirmed the limited presence of 3 (<1%), the expected presence of inactive dibenzofulvene and the total absence of diketopiperazine.

At this point the aqueous radioactive solution of 4 was divided in two parts, which were used as follows.

Preparation of Pyroglutamic-leucine-[3,4,5-^{3}H(N)]-tryptophan-methylester [Pyr-*Leu-TrpOMe] (Pol-509) 5.

Half of solution containing 4 (31.4 mg, 0.085 mmol, total activity: 399 GBeq) was lyophilized and 3 ml of CH_3CN were added to the darkish residue, obtaining a brownish opalescent solution. Pyroglutamic acid (12.3 mg, 0.095 mmol), 40 µl (0.289 mmol) of TEA and 39.5 mg (0.1 mmol) of HBTU were added at 0°C and the mixture was stirred for 2.5 hours in the dark at r.t.. Then it was diluted with 2 ml of CH_3CN, 28 ml of H_2O and 4 ml of NaOH 0.01 N and passed through two SPE-columns to block the tripeptide 5. Each SPE-column was washed with 0.5 ml of H_2O which was recovered and its inactivity checked. After this, 5 was recovered eluting each SPE-column with 5 ml of CH_3CN. Radio-HPLC analysis (condition B) showed the high level of purity for product 5 (see Fig. 1). The solution was evaporated under vacuum at r.t. and the residue so obtained was dissolved in 15 ml of water and lyophilized. The tripeptide 5 was recovered as a white-grey solid (35 mg, 0.079 mmol, 93%). Total activity: 364 MBeq and specific activity: 4.60 GBeq/mmol. It was dissolved in physiological solution at the concentration of 0.5 mg/ml, useful for further biological investigations.

Preparation of N-Benzyloxycarbonyl-proline-leucine-[3,4,5-^{3}H(N)]-tryptophan-methylester [CBZ-Pro-*Leu-TrpOMe] 6.

The remaining half solution (31.4 mg, 0.085 mmol, total activity: 399 MBeq) was lyophilized and 3 ml of CH_3CN were added to the darkish residue, obtaining a brownish suspension. At 0°C, 20 mg (0.081 mmol) of N-CBZ-Pro, 50 µl (0.361 mmol) of TEA, 37 mg (0.094 mmol) of HBTU were added and the mixture was stirred for 3 hours in the dark at r.t.. Then it was diluted with 3 ml of CH_3CN, 9 ml of H_2O, 1.350 ml of NaOH 0.01 N and passed through two SPE-columns. Each of these was washed with a mixture of 6 ml

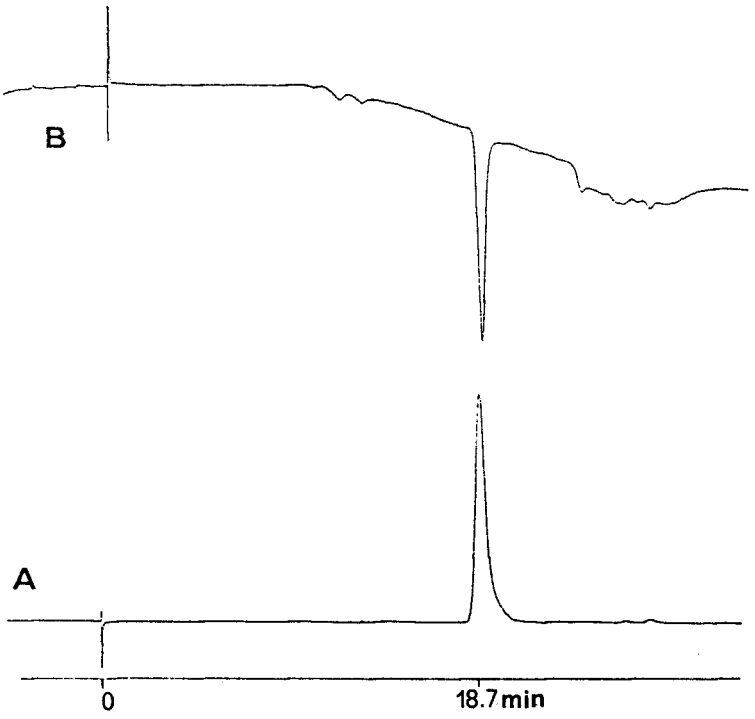


Fig. 1 Radio-HPLC analysis of tritiated [Pyr-*Leu-TrpOMe] 5 (trace A) and corresponding U.V. trace (B).

of CH_3CN and 10 ml of H_2O. The trapped tripeptide 6 and dipeptide 4 residue were unblocked eluting each SPE-columns with CH_3CN (5 ml). Radio-HPLC analysis (condition B) showed the presence of 6 (40 mg, 0,071 mmol, 88%, total activity: 316 MBeq, specific activity: 4,45 GBeq/mmol) with 10% residue 4.

Preparation of N-Benzyloxycarbonyl-proline-leucine-[3,4,5-^{3}H(N)]-tryptophan acid [CBZ-Pro-*Leu-TrpOH] (Pol-443) 7.

The organic solution containing 6 (40 mg, 0.071 mmol), was evaporated at r.t. under vacuum up to 6.5 ml. At 0°C, 5.8 ml of H_2O and 1.3 ml of NaOH 0.1 N were rapidly added and the solution was left in the dark at r.t. for 4.0 hours, diluted with 4.2 ml

of H_2O and passed through two SPE-columns. The alkaline filtered liquid was acidified at pH 3.0 with HCl 0.2 N and the obtained emulsion was passed through other two SPE-columns, trapping 7, that instead was recovered eluiting each SPE-columns with CH_3CN (5 ml). Radio-HPLC analysis (condition B) showed only the presence of 7 (see Fig. 2). The organic solution was concentrated under vacuum at r.t., and the stoichiometric amount of NaOH 0.01 N was added producing a clear solution which was lyophilized. The sodium salt of 7 was recovered (37 mg, 0.064 mmol, 90%). Total activity: 288 MBq and specific activity: 450 GBeq/mmol. It was dissolved in physiological solution at the concentration of 0.9 mg/ml, useful for further biological investigations.

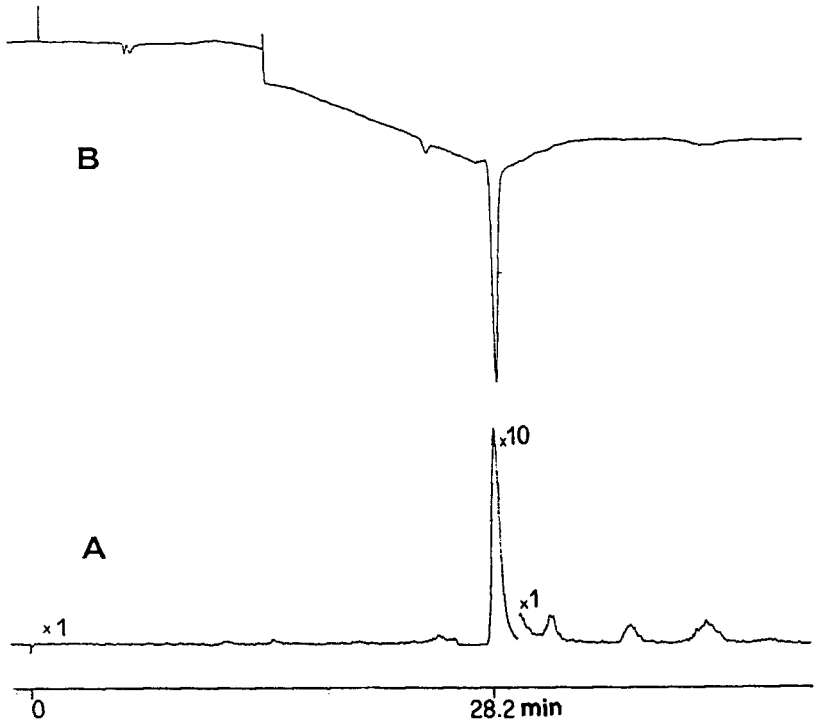


Fig. 2 Radio-HPLC analysis of tritiated [CBZ-Pro-*Leu-TrpOH] 7 (trace A) and corresponding U.V. trace (B).

RESULTS AND DISCUSSION

The proposed reaction pathway (Scheme I) shows one of the many possibilities to reach the tripeptides 5 and 7 starting from tritiated Leu 1. In this pathway we carefully avoided any reagents or synthetic procedures which could give place to racemization processes, and chose suitable reagents which allowed us to carry out simple and fast purification of the products from unreacted starting compounds or expected co-products. These factors, together with the limited overall radiochemical exchange (only 4%), measured during all the reaction led us to consider the proposed reaction pathway the most efficacious for the radiochemical syntheses of the desired products. The tripeptides 5 (POL-509) and 7 (POL-443) were obtained respectively in 70% and 60% yield.

In particular the Scheme I concerns:
i) protection of the aminic group of *LeuOH 1 with the FMOC group and (iii) relative deprotection.

The choice of the succinimidic derivative (26) of the FMOC, used as precursor of the protective group, is essentially due to its simple, fast and complete reaction with the tritiated Leu, producing only the desired products without any formation of FMOC-oligodipeptides (26 d,e), typical of other FMOC derivatives.

Taking into account the elimination process of the protective group, the FMOC group is removed from the peptide as soon as the required basic conditions are reached. This simple behaviour is not peculiar of other classic aminic protective groups, as Boc (tert-Butyloxycarbonyl) or Cbz (Benzyloxycarbonyl) which require more complicated elimination conditions when the indolic ring (tryptophan) is present. In fact, their removal may result in the formation of several collateral by-products (22 b,c). For the Boc group, it is necessary to find the suitable scavengers to avoid the tert-butylation of the indolic ring (27), that can be morover

reduced (28 a,b) during the final deprotective hydrogenolysis of the CBZ group (22 b, 28 c). In addition the FMOC group is more lipophilic with respect to Boc and Cbz groups and in particular with respect to the unprotected peptide. This permits the simple purification and the complete recovery of the peptide using the reverse-phase SPE-technique (25). Moreover, thanks its high U.V. absorbance (23 c), FMOC group permits high sensitive HPLC and TLC analyses(23, 26) so that even low amount of co-products can be evidenced.

ii) Addition of the TrpOMe to the protected tritiated Leu 1.

The formation of this peptidic bond is well favored by equimolar amount of HBTU (24). This reaction is very fast, so as to avoid any partial elimination of the protective group FMOC which could be favored by the free aminic function of the Trp (29). The reaction yield is very high (>93%) and no racemization (24 b) or collateral products are noted. All these characteristics, together with the easy elimination (SPE-technique) of the water soluble HBTU by-products, led us toward its preference with respect to other standard condensing reagents (carbodiimides, mixed anhydrides, etc.)(22 a).

iii) Elimination of the FMOC protective group.

This step consists of the basic DEA (23, 29) degradation of the carbammic function formed by the FMOC group with the aminic function of Leu. An advantage of the use of DEA is its volatility and, more important, its inability to react with the dibenzofulvene, formed as by-product from the FMOC degradation. This side-reaction is peculiar to other aminic compounds, like piperidine (23) and involved complicate purification steps. However, the DEA-deprotection reaction is noted (30) for another collateral process concerning the intra molecular cyclization induced by too

long-time basic reaction condiction and producing a diketopiperazine (radioactive in this case). Apart from the loss of the radioactivity, which this undesired compound implies, it should be necessary the application of sophisticated separative methods (like long and low-yielding HPLC collections), even when small amounts of diketopiperazine happens.

However the correct choices of the DEA quantity, temperature, reaction time, solvent (CH_3CN) and dilution level allowed us to avoid the collateral process above described.

An important point concerns the complete evaporation of DEA before the water addition. In fact its minimum presence in aqueous solution should induce the fast complete hydrolisis of the ester function of **4**, which instead has been obtained in 98% yield, without any racemization products.

iv) Addition of Pyr to tritiated **4**.

The employement of HBTU favours the peptidic bond synthesis and permits to obtain **5** (POL-509) in 93% yield. The excess of Pyr does not produce any by-products and the residue amount can be easily eliminated by a simple step of SPE procedure.

v) Addition of CBZ-Pro to tritiated **4**.

Also in this case the employment of the HBTU has allowed the formation of the peptidic bond in a very simple and clean way. However here, CBZ-Pro has been used in sub-stoichiometric amount. In fact, preliminary blank runs showed the presence of difficulty eliminable by-products when the reaction was carried out using greater amount of CBZ-Pro.

vi) Hydrolisis of the ester function of **6** with formation of **7**.

The usual alkaline hydrolisis (MeOH/NaOH) (22 b) has not been applied to **6**, since considerable racemization of **7** was

observed during several blank runs (31). A particular basic hydrolisis, specific for this reaction step, has been employed as reported in the experimental part. In these conditions, with proper adjustment of the pH values during the reaction, the acidic tripeptide 7 (POL-443) is recovered in good yield without any racemized product.

From the radiochemical point of view we have to point out the small decrease (<4%) of the specific activity during all the proposed pathway, notwithstanding the numerous isotopic exchange possibilities induced by the drastic acid and basic conditions which have been used in several synthetic steps. This advantage is essentially due to the hydrocarbon nature of the tritium atoms which are exclusively located in the isobutyl group of the starting tritiated leucine 1.

At last, a particular comment must be done for the employment in this work of the reverse-phase solid phase extraction technique. The correct choice of the reagents and experimental conditions allow to carry out simple, rapid, efficient and complete purifications of the desired products, permitting "one pot" massive recovering of the final products (up to 100 mg). In particular, in this work, where radioactive substances at high specific activity have been used, we could verify the reliability of this technique, recovering completely the radioactive products passed through the SPE-columns and confirming the minimum absorption when these columns were eluted with the appropriate solvents.

ACKNOWLEGDEMENT

The authors acknoledge with gratitude Mrs. G. Naruli for the preparation of the manuscript.

REFERENCES

1) Abbreviations used for amino acids follow the rule of the IUPAC-IUB Commission of Biochemical Nomenclature in J. Biol. Chem. 247: 977 (1972). All the amino acids are of the "L"-configuration, that was omitted for semplicity.

2) Bieber A.L. - Handbook of Experimental Pharmacology 52: 295 (1979).

3) a) Ferreira S.H. - Brit. J. Pharmacology 24: 163 (1965). b) Ondetti M.A.. Williams N.J., Sabo E.F., Pulsec J., Weaver E.R., Kocy O. - Biochemistry 10: 4033 (1971).

4) Politi V., De Luca G., Di Stazio G., Schinina' E., Bossa F. - Peptides 6 (suppl. 3): 343 (1985).

5) a) Annual Drug Data report 10: 791 (1990). b) Mattei M., Gorini A., Lavaggi M.V., Sumerska T., Politi V., Colizzi V. - J. Chemotherapy III (suppl. 3 - June): 160 (1991).

6) Angelini G., Carnevaletti F., Piccinini F. - J. Lab. Compds. Radioph. XXXI: 289 (1992).

7) a) Angelini G., Speranza M., Felici M, Luna M. - J. Radioan. Nucl. Chem. Lett. 87: 283 (1984). b) Speranza M., Giovannozzi-Sermanni G., Angelini G., Artemi F., Badiani M. - Agric. Ital. 67 (1985). c) Speranza M., Luna M., Angelini G., Artemi F., Badiani M. - Agric. Ital. 77 (1985).

8) Cellai L., Colosimo M., Marchi E. - J. Lab. Compds. Radioph. XI: 1287 (1983).

9) Morgat J.L., Fromageot P. - in "Proceeding of Symposium on new developments in radiopharmaceuticals and labelled compounds" Copenhagen, 26-30 March, 1973.

10) Angelini G., Segre A.L., Speranza M., Altman J. - J. Org. Chem. 45: 3291 (1980).

11) Angelini G., Keheyan Y., Lilla G., Segre A.L. - Gazz. Chim. It. 119: 107 (1989).

12) Angelini G., Lilla G., Sparapani C., Ursini O., Rossi E., Segre A.L., McAllister M.A., Tidwell T.T. - Can J. Chem. (in press).

13) a) Cacace F., Speranza M. in J.M. Farrar and W.H. Saunders Jr., Eds. "Techniques for the Study of Ion-molecule Reactions", Wiley, New York, Chapter VI, (1988). b) Cacace F. - Science 250: 392, (1990).

14) Van Nispen J.W., Bijl W.A.A.J., Hendrix A.M.M., Greven H.M. Rec. Trav. Chim. Pays-Bas 102: 276 (1983) and ref. therein.

15) Hardy P.M., Sheppard P.W., Brundish D.E., Wade R. - J. Chem. Soc. Perkin Trans. I: 731 (1983) and ref. therein.

16) a) Labbe'-Jullie C., Blanot D., Morgat J.L., Kitabgi P., Checler F., Vincent J.P., Graniez C., Van Rietschoten J. - Biochimie 65: 553 (1983). b) Sasaki A.N., Michelot R., Morgat J.L., Genet R., Potier R. - Int. J. Pept. Prot. Res. 27: 366 (1986).

17) Brundish D.E., Wade R. - J. Lab. Compds. Radioph. XXIII: 9 (1985) and ref. therein.

18) Parnes H., Shelton E.J.- Int. J. Pept. Prot. Res 27: 239 (1986).

19) Parnes H., Shelton E.J. - J. Lab. Compds. Radioph. XXI: 263 (1984) and ref. therein.

20) Hellio F., Lecocq G., Morgat J.L., Gueguen P. - J. Lab. Compds. Radioph. XXVIII: 991 (1990) and ref. therein.

21) For a similar approach: a) du Vigneaud V., Schnelder C.M., Stouffer J.E., Murti V.V.S., Arosak J.P., Winestock G. - J. Am. Chem. Soc. 84: 409 (1962). b) Sjöholm I., Carlsson L. - J. Labelled Compds. 3: 3 (1967).

22) a) "The Peptide" Analysis, Synthesis and Biology. E. Gross, J. Meienhofer Eds. Acad. Press. N.Y. Vol 1° "Major Method of Peptide Bond Formation" (1979). b) ibid. Vol. 3° "Protection

of Functional Groups in Peptide Synthesis" (1981). c) Bodanszky M., Martinez J. - Synthesis 333 (1981).

23) a) Carpino L.A., Han G.Y., - J. Am. Chem. Soc. 92: 5748 (1970). b) Carpino L.A., Han G.Y., - J. Org. Chem. 37: 3404 (1972); ibidem 38: 4218 (1973). c) Carpino L.A. - Accs. Chem. Res. 20: 401 (1987) and ref. therein.

24) a) Dourtoglou V., Ziegler J.C., Gross B. - Tetrahedron Letts. 1269 (1979). b) Dourtoglou V., Gross B., Lambropoulou V., Zioudrou C. - Synthesis 572 (1984). c) Knorr R., Trzenak A., Bannwarth W., Gillesen D. - Tetrahedron Letts. 30: 1927 (1989).

25) a) "Solid Phase Extraction for sample preparation" M. Zief, R. Kiser, J.T. Baker. Inc (1988) and ref. therein. b) "Baker-10-SPE(TM)" Application Guide Vol 1° (1982) J.T. Baker Inc. c) "Baker-10-SPE(TM)" Application Guide Vol 2° (1984) J.T. Baker Inc.

26) a) Paquet A. - Can J. Chem. 60: 976 (1982). b) Lapatsanis L., Milias G., Froussios K., Kolosov M. - Synthesis 671 (1983). c) Field G.B., Noble R.L.- Int. J. Pept. Prot. Res. 35: 161 (1990). d) Sigler G.F., Fuller W.D., Chaturvedi N.C., Goodman M., Verlander M. - Biopolymers 22: 2157 (1983) and ref. therein. e) Tessier M., Albericio F., Pedrosa E., Grandas A., Eritjia R., Giralt E., Van Rietschoten J. - Int. J. Pept. Prot. Res. 22: 125 (1983).

27) a) Jaeger E., Thamm P., Wünsch E. - Hoppe-Seyler's Physiol. Chem. 359: 1629 (1978). b) Löw M., Kisfaludy L., Sohar P. - Hoppe-Seyler's Physiol. Chem. 359: 1643 (1978).

28) a) Rebek J., Tai D.F., Shue Y.K. - J. Am. Chem. Soc. 106: 1813 (1984). Kisfaludy L., Korenczki F., Mohácsi T., Sajgo M., Fermandjian S. - Int. J. Pept. Prot. Res. 27: 440 (1986) and ref. therein. b) Mery J., Calas B. - Int. J. Pept. Prot.

Res. 31: 412 (1988). and ref. therein. Kikugawa Y., Kashimura M. - Synthesis 785 (1982). c) El Amin B., Anantharamaih G.M., Royer G.P., Means G.E. - J. Org. Chem. 44: 3442 (1979). Coleman D.R., Royer G.P. - J. Org. Chem. 45: 2268 (1980). Anwer K.M., Spatola A.F. - Synthesis 929 (1980) and ref. therein.

29) Bodanszky M., Deshmane S.S., Martinez J. - J. Org. Chem. 44: 1622 (1979).

30) Bodanszky A., Bodanszky M., Chandramouli N., Kwei J.E., Martinez J., Tolle J.C. - J. Org. Chem. 45: 72 (1980).

31) Margonelli A.- unpublished results.