

## Synthesis of a [ $^{14}\text{C}$ ]-labelled Photoactivatable Cyclic Tetrapeptide: [carbonyl- $^{14}\text{C}$ ]-4-benzoylbenzoyl MeSer<sup>1</sup>-tentoxin.

J.-M. Gomis<sup>a\*</sup>, J. Santolini<sup>b#</sup>, F. Cavelier<sup>c</sup>, J. Verducci<sup>c</sup>, E. Pinet<sup>bs</sup>, F. André<sup>b</sup>, J.-P. Noel<sup>a</sup>.

<sup>a</sup>CEA/Saclay, Département de Biologie Cellulaire et Moléculaire, Service des Molécules Marquées, Bât. 547, 91191 Gif/Yvette cedex, France.

<sup>b</sup>CEA/Saclay, Département de Biologie Cellulaire et Moléculaire, Section de Bioénergétique, 91191 Gif/Yvette cedex, France.

<sup>c</sup>Laboratoire des Aminoacides, Peptides et Protéines (LAPP), UMR 5810 CNRS, Université Montpellier II, 34095 Montpellier cedex 5, France.

### SUMMARY

The synthesis of a new photochemical probe for labelling tentoxin **1** binding sites in the soluble part of chloroplast  $F_0F_1$  ATPsynthase is described. [Carbonyl- $^{14}\text{C}$ ]-4-benzoylbenzoyl MeSer<sup>1</sup>-tentoxin **6** was synthesised by coupling MeSer<sup>1</sup>-tentoxin **2** with [carbonyl- $^{14}\text{C}$ ]-4-benzoylbenzoic acid **3**, itself obtained by coupling [ $^{14}\text{C}$ ]-benzoyl chloride with the dilithio derivative of 4-bromobenzoic acid (**9**). Photolysis of the probe in methanolic solution occurs readily upon irradiation with UV light at 366 nm.

**key-words:**  $F_0F_1$ ,  $\text{H}^+$  ATPase, chloroplast, cyclopeptide, photolabelling, tentoxin, ( $^{14}\text{C}$ )-benzoylbenzoic acid.

<sup>#</sup> fellow from the Ministère de l'Éducation Nationale, de la Recherche et de la Technologie, grant No 96-5-11984.

<sup>s</sup> fellow from the Commissariat à l'Énergie Atomique, present address: Organogel Canada Ltd, 1400 Bd du Parc Technologique, Québec, G1P 4R7, Canada.

\* to whom correspondence should be addressed:

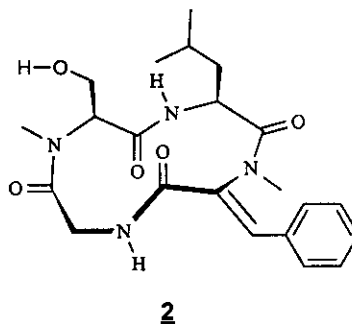
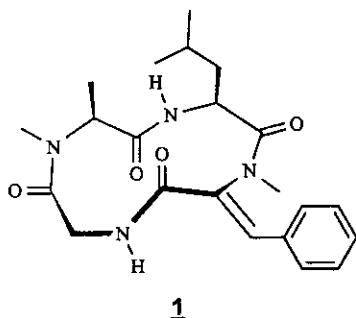
Jean-Marie GOMIS, DBCM/SMM, CEA-Saclay, 91191 Gif-sur-Yvette cedex, France.

Tel: (0033) 1 69 08 24 63, Fax: (0033) 1 69 08 79 91, Email: jean-marie.gomis@cea.fr

## INTRODUCTION

Tentoxin (TTX) **1** is a natural cyclic tetrapeptide [*cyclo*-(L-MeAla<sup>1</sup>-L-Leu<sup>2</sup>-MePhe[(Z) $\Delta$ ]<sup>3</sup>-Gly<sup>4</sup>)] secreted by a phytopathogenic fungus *Alternaria alternata* (1,2). *In vitro* tentoxin binds to the F<sub>1</sub> moiety of chloroplast F<sub>0</sub>-F<sub>1</sub> H<sup>+</sup>-ATPase (CF<sub>1</sub>). The ATPase activity of CF<sub>1</sub> is inhibited by low concentrations of tentoxin (about 10<sup>-8</sup> M), while this activity is stimulated at higher concentrations (10<sup>-5</sup> M).

Equilibrium dialysis experiments with CF<sub>1</sub> from spinach chloroplasts using [[methyl-<sup>14</sup>C]-MePhe[(Z) $\Delta$ ]<sup>3</sup>-tentoxin revealed the presence of two major binding sites of distinct affinities (3), and it was suggested that the sequential binding of two molecules of tentoxin account for its double effect on CF<sub>1</sub> ATPase activity. However, the location of the two binding sites in the CF<sub>1</sub> structure is still unknown (4).



Photolabelling reagents are commonly used to characterise ligand binding sites in enzymes by photochemical labelling. Among several photolabelling reagents, benzophenone derivatives offer some advantages (5). They are remarkably stable chemically and react with C-H bonds to form covalent bonds.

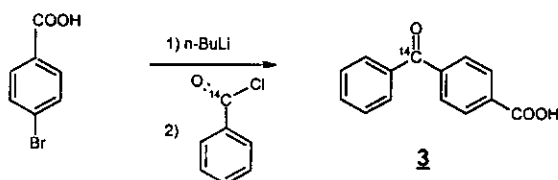
In this report, the synthesis of [carbonyl-<sup>14</sup>C]-4-benzoylbenzoyl-[*cyclo*-(L-MeSer<sup>1</sup>-L-Leu<sup>2</sup>-MePhe[(Z) $\Delta$ ]<sup>3</sup>-Gly<sup>4</sup>)] or [carbonyl-<sup>14</sup>C]-4-benzoylbenzoyl-MeSer<sup>1</sup>-TTX **6** is described. This photoactivatable derivative of tentoxin was designed to specifically inhibit the ATPase activity of CF<sub>1</sub>, and thus to characterise the high affinity binding site of TTX in CF<sub>1</sub> (4).

## RESULTS AND DISCUSSION

The radioactive photolabelling reagent, the benzophenone photoreactive group, can be introduced into a molecule *via* radioactive 4-benzoylbenzoic acid. Natural tentoxin has no available chemical function to couple this reagent. We therefore initially prepared various functionalised analogues of TTX (6). For the introduction of the radioactive photoreactive

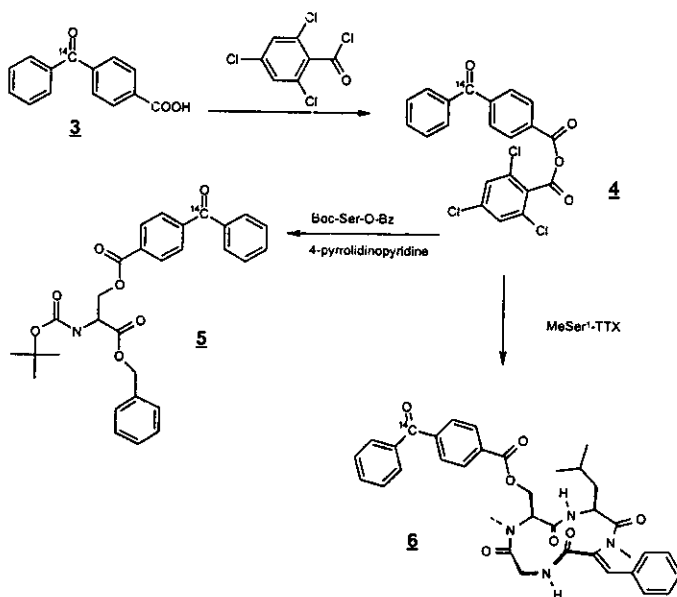
group, we selected *cyclo*-(L-*N*-MeSer<sup>1</sup>-L-Leu<sup>2</sup>-*N*-MePhe[(Z)Δ]<sup>3</sup>-Gly<sup>4</sup>) **2** (MeSer<sup>1</sup>-TTX), an analogue of tentoxin where L-*N*-Me-Alanine was replaced by L-*N*-Me-Serine (**7**, **8**). The side chain alcohol of serine was then used to introduce the radioactive 4-benzoylbenzoic acid through an ester function.

Synthesis of [<sup>14</sup>C-carbonyl]-4-benzoylbenzoic acid **3** in 57% yield from (<sup>14</sup>C-carboxy)-benzoic acid was achieved by coupling [1-<sup>14</sup>C]-benzoyl chloride with the dilithio derivative of 4-bromobenzoic acid according to *Scheme 1* (**9**)



Scheme 1

Pure MeSer<sup>1</sup>-TTX was available in small amounts, and to prevent any waste of this synthetic analogue we first tested the reaction of 4-benzoylbenzoic acid with serine side chain, using Boc-Ser-OBz as starting material and we considered the inactive tetrapeptide as the most valuable product. Results obtained in our laboratory (**10**) prompted us to perform the coupling with the mixed anhydride **4** of 4-benzoylbenzoic acid and 2,4,6-trichlorobenzoic acid using 4-pyrrolidinopyridine **8** (**11**) (*Scheme 2*). The expected product [carbonyl-<sup>14</sup>C]-4-benzoylbenzoyl-Boc-Ser-OBz **5** was obtained in 45% radioactive yield and 90% chemical

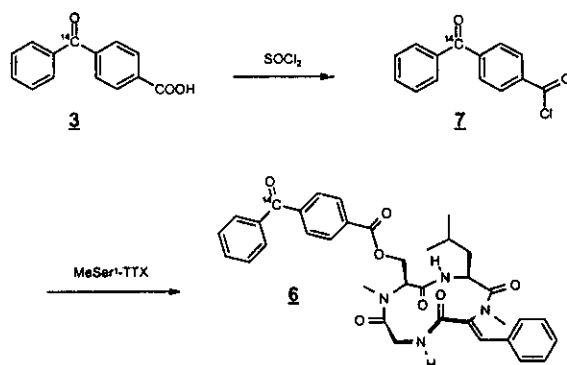


Scheme 2

yield from the protected L-serine using two equivalents of mixed anhydride **4**. However, the same reaction of mixed anhydride **4** with MeSer<sup>1</sup>-TTX did not lead to the expected condensation (Scheme 2).

Once we obtained the desired product by the method described later, we demonstrated that this lack of condensation was due to the purification technique used for MeSer<sup>1</sup>-TTX. When we used reversed phase chromatography and methanol-water as the mobile phase, the condensation failed due to some residual water being present even after one night under vacuum. In contrast if we used acetone for the purification we obtained the expected product in a quantitative chemical yield. To complete this study, we synthesised a new batch of MeSer<sup>1</sup>-TTX and synthesised radioactive [carbonyl-<sup>14</sup>C]-4-benzoylbenzoyl-MeSer<sup>1</sup>-TTX **6** using only 1.3 equivalent of mixed anhydride. In this case (purification with acetone and 1.3 eq of mixed anhydride) the radioactive yield was 77% and the chemical yield quantitative.

After the failure of the first synthesis of the mixed anhydride purified with water, the condensation was achieved with the 4-benzoylbenzoyl chloride as displayed in Scheme 3. Condensation of 1.3 eq of [carbonyl-<sup>14</sup>C]-4-benzoylbenzoyl chloride **7** with MeSer<sup>1</sup>-TTX provided the desired [carbonyl-<sup>14</sup>C]-4-benzoylbenzoyl-MeSer<sup>1</sup>-TTX **6** in 65 % chemical yield and 50% radioactive yield after liquid chromatography purification.



Scheme 3

The [carbonyl-<sup>14</sup>C]-4-benzoylbenzoyl-MeSer<sup>1</sup>-TTX compound showed characteristic UV absorption bands at ca. 260 nm, with a shoulder at 281 nm, characteristic of *N*-MePhe[(*Z*) $\Delta$ ] residue absorption, and also at 225 nm (Figure 1, spectrum a). Upon irradiation of a methanolic solution of **6** with monochromatic UV light ( $\lambda=366$  nm), the benzophenone band at 260 nm decayed with a half life of around 100 s in our photolysis setup (Figure 1). A new band corresponding to the isomer produced by photoisomerization appeared at around 240 nm, and a characteristic isosbestic point is evident at 250 nm.

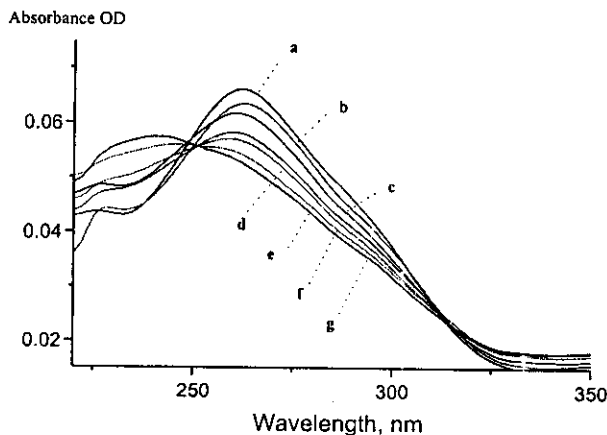


Figure 1:

Photolysis of [carbonyl- $^{14}\text{C}$ ]-4-benzoylbenzoyl-MeSer<sup>1</sup>-TTX (UV spectra).

UV absorption spectra of compound **6** recorded in the photolysis reaction mixture at the following times: a)  $t = 0$  s (reference UV spectrum of compound **6**), b)  $t = 15$  s, c)  $t = 75$  s, d)  $t = 120$  s, e)  $t = 240$  s, f)  $t = 900$  s, g)  $t = 1800$  s. The sample concentration was  $5 \mu\text{M}$ , and was irradiated with monochromatic UV light ( $\lambda = 366$  nm,  $W = 0.3$  mW/cm<sup>2</sup>) in methyl alcohol at  $25^\circ\text{C}$ .

## EXPERIMENTAL

### General

[ $^{14}\text{C}$ ]-Barium carbonate, used for the standard synthesis of [carboxyl- $^{14}\text{C}$ ]-benzoic acid, was obtained from Slavia. Reagents were from Aldrich. Solvents were distilled with appropriate desiccant reagents and kept over molecular sieves. HPLC analyses were carried out on a Merck L 6200 system. Radioactive monitors were models LB 505 and LB 503 from Berthold. The UV detector was a Merck system, model L 4250.  $^1\text{H}$  NMR spectra were recorded on a Bruker AM 300 spectrometer operating at 300.13 MHz. Radio-TLC is recorded using the Berthold system, model LB 2821. Specific activities were determined on a Finnigan mass spectrometer, model 4600.

### [Carbonyl- $^{14}\text{C}$ ]- 4-benzoylbenzoyl-Boc-L-serine-OBz **5**.

14.1  $\mu\text{L}$  of 2,4,6-trichlorobenzoyl chloride was added to 0.1 mCi (0.1 mmol, SA 1 mCi/mmol) of [carbonyl- $^{14}\text{C}$ ]-4-benzoylbenzoic acid dissolved in 1 mL of THF with 15.3  $\mu\text{L}$  of TEA. The solution was stirred for 15 h and then evaporated to dryness and dissolved in 5

mL of dry benzene. 15 mg (0.05 mmol) of Boc-L-Ser-OBz and 16.3 mg (0.11 mmol) of 4-pyrrolidinopyridine were added and the solution was stirred at room temperature for 3 h. The organic solution was diluted with ethyl acetate (40 mL), washed with 5% aqueous citric acid (5 mL), with saturated bicarbonate (5 mL), and then with water up to pH 7.

The product was purified on a silica gel column with hexane, acetone (70:30). 0.045 mCi of pure product were obtained (radioactive yield 45% and chemical yield from Boc-L-Ser-OBz 90%). The mass spectra was in agreement with the expected product. TLC analysis on silica gel with hexane, acetone (80:20) showed one spot of R<sub>f</sub> 0.27. Mass spectrometry (CI/NH<sub>3</sub>) of [carbonyl-<sup>14</sup>C]-4-benzoyl-Boc-L-serine-OBz **5**: m/z+18=521.

**[Carbonyl-<sup>14</sup>C]- 4-benzoylbenzoyl MeSerTTX **5** (scheme 2).**

(MeSerTTX was previously purified by chromatography using methanol-water)

12.4 μL of 2,4,6-trichlorobenzoyl chloride was added to 4.76 mCi (0.088 mmol) of [carbonyl-<sup>14</sup>C]-4-benzoylbenzoic acid dissolved in 1 mL of THF with 13.5 μL of TEA. The solution was stirred for 15 h and then evaporated to dryness and dissolved in 5 mL of dry benzene. 19.7 mg (0.046 mmol) of MeSerTTX (previously purified by reversed phase chromatography with methanol-water and dried for one night under vacuum), 15 mg (0.10 mmol) of 4-pyrrolidinopyridine and 13.5 μL of TEA were added and the solution was stirred at room temperature for 15 h. The organic solution was diluted with ethyl acetate (40 mL), washed with 5% aqueous citric acid (5 mL), with saturated bicarbonate (5 mL), and then with water up to pH 7.

We did not obtain the expected product. 9 Mg of MeSerTTX was recovered after purification by liquid chromatography (silica gel column with acetone) for the following radioactive synthesis.

**[Carbonyl-<sup>14</sup>C]- 4-benzoylbenzoyl MeSerTTX **6** (scheme 2).**

(MeSerTTX was previously purified by chromatography using acetone)

6 μmoles (100 μL of a 1 mL THF solution containing 9.38 μL of 2,4,6-trichlorobenzoyl chloride) of 2,4,6-trichlorobenzoyl chloride was added to 330 μCi (6 μmol) of [carbonyl-<sup>14</sup>C]-4-benzoylbenzoic acid dissolved in 1 mL of THF with 6.6 μmol of TEA (100 μL of a 1 mL THF solution containing 9.2 μL of TEA). The solution was stirred for 12 h at room temperature and then evaporated to dryness. 2 mg (4.65 μmol) of MeSerTTX (previously purified by normal phase chromatography with acetone and dried for one night under vacuum) in 1 mL of anhydrous benzene, 6.6 μmol of 4-pyrrolidinopyridine (100 μL of 1 mL anhydrous

benzene solution containing 9.8 mg of 4-pyrrolidinopyridine) and 6.6 μmol (100 μL of a 1 mL anhydrous benzene solution of 9.2 μL of TEA) were added and the solution was stirred at room temperature for 15 h. The organic solution was diluted with ethyl acetate (40 mL), washed with 5% aqueous citric acid (5 mL), with saturated bicarbonate (5 mL), and then with water up to pH 7.

The products were analysed by TLC (silicagel acetone-hexane 50/50) Rf 0.63

MeSerTTX was totally consumed and the radiochemical purity was 77 %. The radioactive impurity was [carbonyl-<sup>14</sup>C]-4-benzoylbenzoic acid.

### [Carbonyl-<sup>14</sup>C]-4-benzoylbenzoyl-MeSerTTX **6** (scheme 3)

2 ML of thionyl chloride was added to 1.5 mCi (0.028 mmol, 1.3 eq) of [carbonyl-<sup>14</sup>C]-4-benzoylbenzoic acid and the solution was refluxed for 2 h. The solution was evaporated to dryness and the residue was dissolved in anhydrous benzene and evaporated again to dryness. 9 Mg (0.021 mmol) of MeSerTTX was added to the previously dried acyl chloride dissolved in 1 mL of anhydrous THF and 10 μL of TEA. The solution was stirred for 15 h at room temperature. The organic solution was diluted with ethyl acetate (40 mL), washed with 5% aqueous citric acid (5 mL), with saturated bicarbonate (5 mL), and then with water up to pH 7.

The product was purified on a silica gel column with acetone. 0.75 mCi of pure product was obtained (chemical yield 65% and radioactive yield 50%). HPLC analysis was done on Zorbax SB C18, using elution with acetonitrile, water (45-55). The purity was 98% (R<sub>t</sub>: 18.5min). TLC on silica gel with acetone, hexane (50-50) showed one spot of Rf 0.63. UV spectrometry analysis in methanol (λ<sub>max</sub>: 205 and 257 nm, λ<sub>min</sub>: 230 nm). <sup>1</sup>NMR (CDCl<sub>3</sub>) δppm: 8.07 (2H, d, J=8.3Hz, H3,3' benzoylbenzoyl); 7.82-7.73 (6H, m, H1,1',2,2' benzoylbenzoyl, CHβ ΔPhe and NH Gly); 7.61 (1H, t, J=7.4Hz, H5 benzoylbenzoyl); 7.48 (2H, dd, J<sub>1</sub>=7.3Hz, J<sub>2</sub>=7.4Hz, H4,4' benzoylbenzoyl); 7.41 (5H, m, Ar. ΔPhe); 5.21 (1H, t, J=21Hz, CHα Gly); 4.91-4.72 (3H, CHα and CH<sub>2</sub>β Ser); 4.14 (1H, m, CHα Leu); 3.65 (1H, d, J=15Hz, CHα' Gly); 3.27 and 3.21 (3H, s, NCH<sub>3</sub> ΔPhe, 2 conformations 10%, 90%); 3.03 and 2.97 (3H, s, NCH<sub>3</sub> Ser, 2 conformations 10% and 90%); 1.62 (1H, m, CHβ Leu); 1.29 (2H, m, CHβ and CHγ Leu); 0.56 (3H, d, CH<sub>3</sub>δ Leu); 0.49 (3H, d, CH<sub>3</sub>δ' Leu).

Mass spectrometry analysis (FAB) of [carbonyl-<sup>14</sup>C]-4-benzoylbenzoyl-MeSerTTX **6**: m/z= 641

### ACKNOWLEDGEMENTS

Thanks are due to Alain Valleix for his support in the analysis of the HPLC data. F. André acknowledges support from the Ministère de l'Éducation Nationale, de la Recherche et de la Technologie, contract ACC-SV5 (interface Chimie-Physique-Biologie) No. 9505221, and from CNRS (grant Physique-Chimie du Vivant, No. 97N21/0122).

### REFERENCES

1. Durbin R.D. and Uchytíl T.F. - *Phytopathology* **67**: 602 (1977)
2. André F. and Pinet E. - *C. R. Soc. Biol.* **191**: 401 (1997)
3. Pinet E., Gomis J.-M., Girault G., Cavelier F., Verducci J., Noël J.-P. and André F. - *FEBS Lett.* **395**: 217 (1996)  
Santolini J., Haraux F., Sigalat C., Moal G. and André F., *J. Biol. Chem.* **274**: 849 (1999)
4. Santolini J., Haraux F., Sigalat C., Munier L. and André F. - *J. Biol. Chem.* **273**: 3343 (1998)
5. Kotzyba-Hibert F., Kapfer I. and Goeldner M. - *Angew. Chem. Int. Ed. Engl.* **34**: 1296 (1995)
6. Cavelier F., Enjalbal C., Santolini J., Haraux F., Sigalat C., Verducci J. and André F. - *Lett. Pept. Sci.* **4**: 283 (1997)
7. Pinet E., Cavelier F., Verducci J., Girault G., Dubart L., Haraux F., Sigalat C. and André F. - *Biochemistry* **35**: 12804 (1996)
8. Cavelier F. and Verducci J. - *Tetrahedron Lett.* **36**: 4425 (1995)
9. Gomis J.-M., Santolini J., André F., Noël J.-P. - *J. Label. Compds. Radiopharm.* *In press*
10. Suzzoni S. (de), Boullais C., Vandais A. and Noël J.-P. - *J. Label. Compds. Radiopharm.* **36**: 739 (1995)
11. Hassner A., Krepski L.R., Alexanian V., *Tetrahedron*, **34**: 2069, (1978)