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High Yield Synthesis of Tentoxin, a Cyclic Tetrapeptide

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Abstract: Tentoxin is a naturally occurring phytotoxic cyclic tetrapeptide excreted by fungi of the *Alternaria alternata* family. The four total syntheses of tentoxin published to date give poor total yields, mainly owing to two difficulties, the introduction of the dehydro amino acid and more especially the cyclization step. Here we describe a method that stereospecifically introduces Z-dehydrophenylalanine (Δ^ZPhe) by a modified Erlenmeyer aldolization reaction. The linear tetrapeptide, Boc-R^1Ala-Leu-$R^2\Delta^Z$Phe-Gly-OMe (R^1, R^2: CH_3, $^{14}CH_3$), the precursor of tentoxin, was obtained in a 72% yield from Boc-Leu-Gly-OH. This linear tetrapeptide, labelled with carbon-14, was used for a comparative study of four cyclization reagents DPPA, DCC–PfpOH, HBTU and HATU. This last was the most effective and gave tentoxin in a 81% cyclization yield. The activated ester formed with this reagent displayed an enhanced capacity for cyclization, permitting cyclization in concentrated medium (10 mM). This new synthetic route gave tentoxin in a 60% yield from Boc-Leu-Gly-OH and offers a means of achieving the synthesis of hitherto elusive analogues. Copyright © 2002 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: cyclization; cyclic peptide; tentoxin; ^{14}C-labelled peptide; dehydro amino acid

INTRODUCTION

Tentoxin (**1**) [1] (Figure 1) is a naturally occurring cyclic tetrapeptide, cyclo-(MeAla-Leu-MeΔ^ZPhe-Gly), produced by several phytopathogenic fungi of the *Alternaria* genus [2,3]. This cyclopeptide is a selective weedkiller that causes the chlorosis of many higher plants [4]. Chlorosis can occur as a consequence of the inhibition of photophosphorylation. Tentoxin specifically inhibits ATP synthesis in the chloroplasts of sensitive species [5] as well as ATP hydrolysis in isolated CF_1[6]. *In vitro* and at low concentrations (10^{-8}–10^{-7} M) tentoxin inhibits F0F1 ATPases, but at higher concentrations it stimulates ATPase activity [7]. To study the mechanism by which tentoxin inhibits CF_1, we developed a synthesis of tentoxin labelled with carbon 14 [8].

Abbreviations: AcOEt, ethyl acetate; ATP, adenosine triphosphate; CF1, chloroplast factor 1; CI-MS, chemical ionization mass spectrum; DCC, dicyclohexylcarbodiimide; DCM, dichloromethane; DCU, dicyclohexylurea; DDQ, dichlorodicyanobenzoquinone; DMAP, dimethylaminopyridine; DMF, *N,N'*-dimethylformamide; DPPA, diphenylphosphoryl azide; EM, effective molarity; ^{1}H-NMR, proton nuclear magnetic resonance; HATU, *N*-[(dimethylamino)-1*H*-1,2,3-triazolo-[4,5-β]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HBTU, *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HOAt, hydroxyazabenzotriazole; HOBt, hydroxybenzotriazole; MeCN, acetonitrile; MeOH, methanol; PfpOH, pentafluorophenol; rt, room temperature; TEA, triethylamine; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TLC, thin layer chromatography

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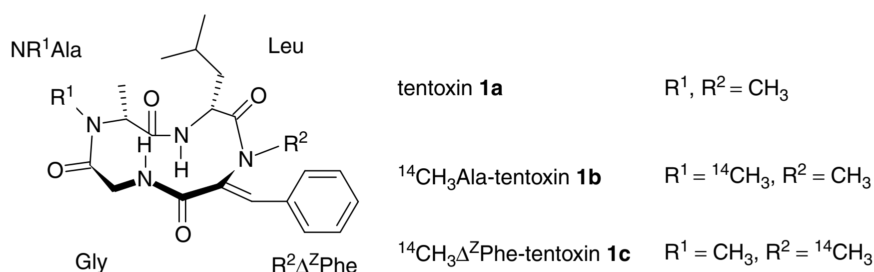

Figure 1 Tentoxin.

In 1997, Delaforge *et al.* [9] showed that tentoxin is metabolized by P450-3A and the two main metabolites formed correspond to the products of mono *N*-demethylation (of tentoxin and isotentoxin). This transformation takes place by a mechanism of oxidative demethylation via an unstable *N*-hydroxymethyl intermediate. To carry out a detailed study of this *N*-demethylation, it was necessary to develop the synthesis of tentoxin labelled at the two *N*-methylation positions: $^{14}CH_3$Ala-tentoxin (**1b**) and $^{14}CH_3\Delta^Z$Phe-tentoxin (**1c**) (Figure 1).

The accessible analogues of tentoxin are difficult to synthesize and so are few in number. The structure/function study of the two preceding proteins has therefore remained limited.

The first synthesis of tentoxin was carried out by Rich and Mathiaparanam [10,11] in 1974. The authors obtained tentoxin by cyclization of the tetrapeptide H-MeAla-Leu-MeΔ^ZPhe-Gly-OH. Jacquier and Verducci [12] in 1984, Edwards *et al.* [13] in 1986 and Cavelier and Verducci [14] in 1995, have proposed other methods to obtain the dehydro amino acid as well as different methods of cyclization. Unfortunately, the overall yields of these various routes do not exceed 20%. The difficulty of introducing the dehydro residue with the correct Z configuration and the difficult cyclization are responsible for these poor results. The development of a new method, which would overcome these two difficulties and offer a high flexibility of transfer of the various residues, is essential for completing the structure/function studies.

As shown by Rich *et al.* [15,16], tentoxin adopts a *trans-cis-trans-cis* conformation of peptide bonds (Figure 1), the two *N*-methyl groups being engaged in the *cis* peptide bond conformation. The linear tetrapeptide sequence chosen by Rich to carry out cyclization has in its centre the Leu-MeΔ^ZPhe peptide bond, which in tentoxin adopts a *cis* conformation. The energy difference between the *cis* and *trans* conformations of the *N*-methylated peptide bonds is small [17]. Thus the Leu-MeΔ^ZPhe peptide bond can easily adopt a *cis* conformation inducing in the linear tetrapeptide a structure close to the cyclized product. Also, the choice of this sequence makes it possible to obtain a sufficiently reactive *C*-terminal function with no risk of epimerization during cyclization.

Here we describe (i) an efficient method for introducing the dehydro amino acid, (ii) a comparative study of the various coupling reagents and (iii) an optimization of the cyclization using HATU. These improvements enabled us to carry out a total synthesis with a yield from Boc-Leu-Gly-OH close to 60% (Scheme 1).

MATERIALS AND METHODS

General

^{1}H-NMR spectra were recorded on a Bruker AM 300 spectrometer operating at 300.13 MHz. The NMR data of tentoxin and intermediates are shown in Table 1. Radio-TLC was recorded using the Berthold system, model LB 2821. Specific activities were determined on a Finnigan mass spectrometer, model 4600. HPLC reverse phase analyses were carried out with a Waters system consisting of a Waters 717 Autosampler, Waters 600 controller, a Waters 486 Tunicate Absorbance Detector and a Packard 150TR Flow scintillation radioactive detector (scintillation cell of 0.6 ml) operating at a flow rate of 1 ml in scintillation liquid (ULTIMA-FLO AP Packard). This system was controlled by the software Millenium. Separation was achieved with a column of Hypersil Elite C18 (150 × 4.6 mm), at a flow rate of 1 ml/min with an eluant gradient of A [90:10 (vol:vol) H_2O/MeCN] and B [10:90 (vol:vol) H_2O/MeCN] under the following conditions: 100% A for 3 min, a linear gradient of 0 to 70% B for 39 min, 30% A and 70% B between 42 and 45 min and

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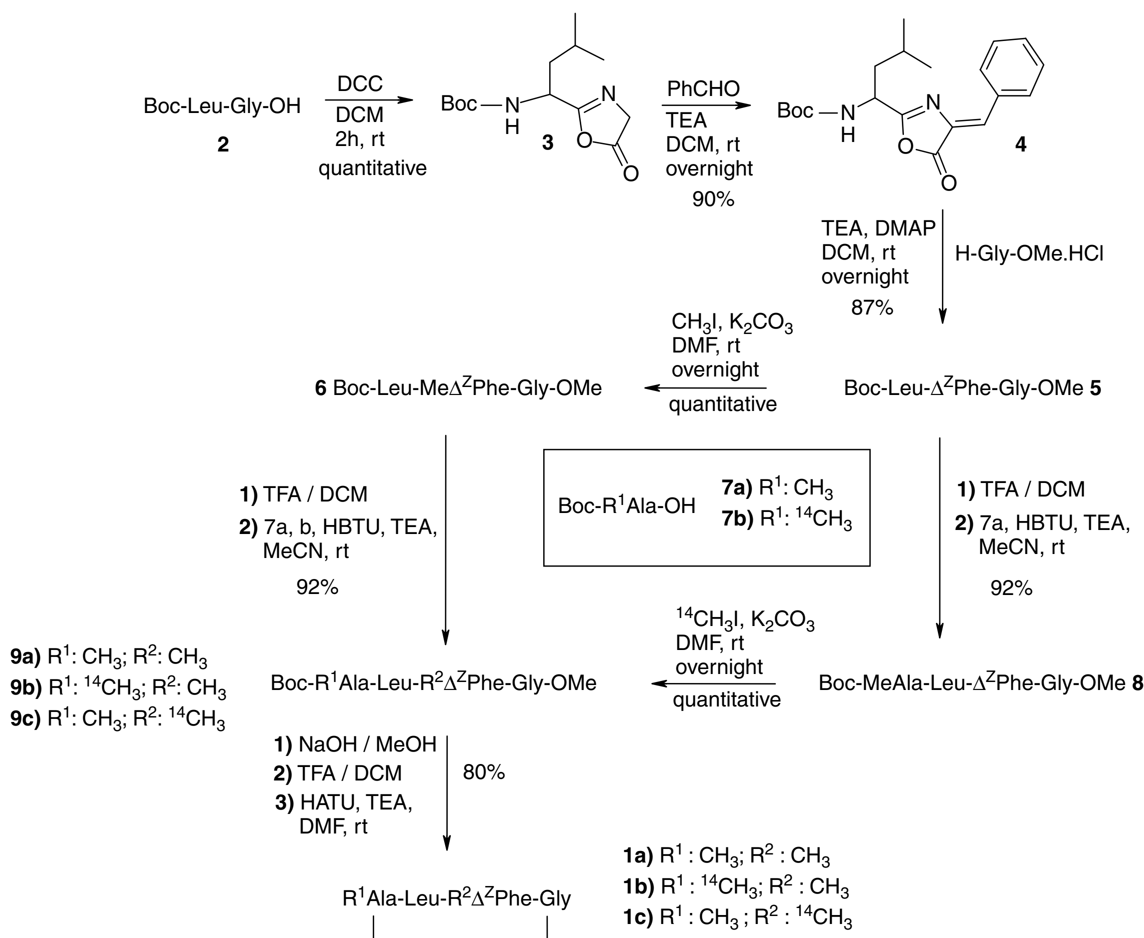

Scheme 1 Synthesis of Tentoxin, $^{14}CH_3$Ala-tentoxin and $^{14}CH_3\Delta^Z$Phe-tentoxin.

then returning to 100% A for 3 min. UV detection operated at 280 nm.

General Protocol of N-Boc Deprotection

To a solution of peptide in 2 ml of DCM, 1 ml of TFA was added. The mixture was stirred at room temperature for 15 min and the solvent was removed. 5 ml of DCM was added to the resulting crude product, which was stirred and evaporated (twice). 10 ml of acetone–heptane (1 : 1) was then added and evaporated to obtain the *N*-deprotected peptide as a white powder in quantitative yield.

General Protocol of O-Me Deprotection

To a solution of peptide in 6 ml of MeOH–H_2O (2 : 1), 4 eq of sodium hydroxide was added. The mixture was stirred at room temperature for 30 min, and the MeOH was removed. The aqueous solution was acidified to pH4 with 0.2 M HCl and then extracted three times with 20 ml of AcOEt. The pH of the aqueous phase must remain below 6. The organic phase was washed with water to neutral pH and evaporated. The resulting residue was dissolved in 10 ml of acetone–heptane (1 : 1) and the solvent was removed to obtain the O-Me deprotected peptide as a white powder in quantitative yield.

Synthesis of Boc-Leu-Gly Oxazolone (3)

To a solution of 1.446 g (5.01 mmol) of Boc-Leu-Gly-OH (**2**) in 25 ml of DCM, 1.02 g (4.99 mmol) of DCC was added. The mixture was stirred at room temperature under an inert atmosphere for 2 h. After filtration of DCU, the solvent was removed under vacuum. The resulting crude residue was dissolved in AcOEt and filtered again to eliminate DCU. The solution was concentrated and dried in

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Table 1 NMR Data of Tentoxin and Intermediates

	AA	NH[NMe]	H_α	H_β	H_γ	H_δ	Others
3 (Boc, Leu, Gly)	Leu	5.15 (d) J^3NH-H_α 8.41 Hz	4.51 (br)	1.5–1.7 (br)	1.5–1.7 (br)	0.874 (d) J^3H_δ-H_γ 6.47 Hz	1.35 H_{Boc}, (s)
	Gly		4.11 (s)				
4 (Boc, Leu, ΔPhe)	Leu	4.98 (d) J^3NH-H_α 8.73 Hz	4.74 (br)	Hβ 1.6–1.8 (br) Hβ' 1.3 (br)	1.6–1.8 (br)	0.97 (d) J^3H_δ-H_γ 6.23 Hz	1.44 H_{Boc} (s)
	ΔPhe			7.17 (s)		8.07 (br)	7.42 $H_{\varepsilon,\varepsilon',\zeta}$ (br)
5 (Boc, Leu, ΔPhe, Gly)	Leu	5.24 (d) J^3NH-H_α 5.38 Hz	4.14 (m)	1.57–1.67 (br)	1.45–1.54 (br)	Hδ 0.899 (d) J^3H_δ-H_γ 5.80 Hz Hδ' 0.953 (d) $J^3H_{\delta'}$-H_γ 5.86 Hz	1.40 H_{Boc} (s)
	ΔPhe	7.56 (s)		7.43 (s)		7.43 (br)	7.32 $H_{\varepsilon,\varepsilon',\zeta}$ (br)
	Gly	8.10 (s)	Hα 3.93 (dd) J^3H_{NH}-H_α 5.09 Hz $J^2H_{\alpha'}$-H_α 17.68 Hz Hα' 4.14 (m) J^3H_{NH}-$H_{\alpha'}$ 5.87 Hz $J^2H_{\alpha'}$-H_α 17.61 Hz				3.71 H_{OMe}, (s)
6 (Boc, Leu, MeΔPhe, Gly)	Leu	4.788 (d) J^3NH-H_α 6.09 Hz	4.156 (br)	$H_{\beta'}$ 1.278 (br) H_β 0.896 (br)	1.170 (br)	H_δ 0.569 (d) J^3H_δ-H_γ 6.72 Hz $H_{\delta'}$ 0.610 (d) $J^3H_{\delta'}$-H_γ 6.72 Hz	1.406 H_{Boc} (s)
	MeΔPhe	[3.238] (s)		7.747 (s)		7.434 (s)	7.434 $H_{\varepsilon,\varepsilon',\zeta}$ (s)
	Gly	8.386 (s)	4.156 (br)				3.747 H_{OMe}, (s)

(*continued overleaf*)

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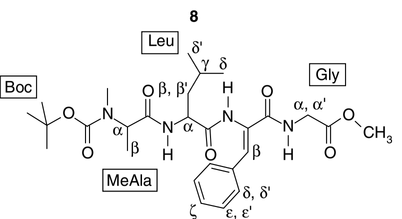
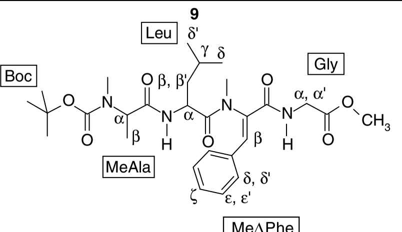
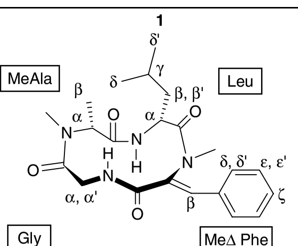
Table 1 (*Continued*)

	AA	NH[NMe]	H_α	H_β	H_γ	H_δ	Others
	MeAla	[2.792] (s)	4.470 (q) J^3H_α-H_β 7.10 Hz	1.316 (d) J^3H_α-H_β 7.13 Hz			1.406 H_{Boc} (s)
	Leu	6.707 (s)	4.301 (br)	$H_{\beta'}$ 1.720–1.805 (br) H_β 1.574–1.688 (br)	1.574–1.688 (br)	H_δ 0.887 (d) J^3H_δ-H_γ 6.10 Hz $H_{\delta'}$ 0.949 (d) $J^3H_{\delta'}$-H_γ 6.30 Hz	
	ΔPhe	7.210–7.365 (large s)		7.394–7.413 (br)		7.394–7.413 (br)	7.210–7.365 $H_{\varepsilon,\varepsilon',\zeta}$ (br)
	Gly	7.945 (s)	4.107 (m)				3.741 H_{OMe}, (s)
	MeAla	[2.666] (s)	4.130 (q) J^3H_α-H_β 6.25 Hz	1.225 (d) J^3H_α-H_β 6.97 Hz			1.441 H_{Boc} (s)
	Leu	7.354 (s)	4.639 (br)	1.209–1.388 (br)	1.209–1.388 (br)	H_δ 0.557 (d) J^3H_δ-H_γ 6.51 Hz $H_{\delta'}$ 0.567 (d) $J^3H_{\delta'}$-H_γ 6.32 Hz	
	MeΔPhe	[3.209] (s)		7.720 (s)		7.407 (s)	7.407 $H_{\varepsilon,\varepsilon',\zeta}$ (s)
	Gly	8.509 (s)	H_α 4.326 (dd) J^2H_α-$H_{\alpha'}$ 10.95 Hz $H_{\alpha'}$ 4.130 (d)				3.709 H_{OMe}, (s)
	MeAla	[2.786] (s)	4.361 (q) J^3H_α-H_β 6.45 Hz	1.530 (d) J^3H_α-H_β 7.19 Hz			
	Leu	7.289 (s)	4.190 (br)	$H_{\beta' Leu}$ 1.583–1.661 (br) $H_{\beta Leu}$ 1.217–1.320 (br)	1.217–1.320 (br)	H_δ 0.485 (d) J^3H_δ-H_γ 5.54 Hz $H_{\delta'}$ 0.605 (d) $J^3H_{\delta'}$-H_γ 6.78 Hz	
	MeΔPhe	[3.173] (s)		7.723 (s)		7.390 (s)	7.390 $H_{\varepsilon,\varepsilon',\zeta}$ (s)
	Gly	8.159 (s)	$H_{\alpha in}$ 5.179 (dd) $J^2H_{\alpha in}$-$H_{\alpha out}$ 14.76 Hz J^3NH-$H_{\alpha in}$ 10.71 Hz $H_{\alpha out}$ 3.558 (d) $J^2H_{\alpha in}$-$H_{\alpha out}$ 15.07 Hz				

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a vacuum. **3** was obtained in quantitative yield as a colourless oil which was used without further purification.

IC-MS (NH4): 271 (56, M+1); 288 (100, M+18). ^{1}H-NMR ($CDCl_3$) δ ppm: 0.874 (2 × $3H_{\delta Leu}$, d, $J^3H_{\delta Leu} - H_{\gamma Leu} = 6.47$ Hz); 1.35 (3 × $3H_{Boc}$, s); 1.5–1.7 (3H, br, $H_{\beta Leu}$, $H_{\beta' Leu}$et $H_{\gamma Leu}$); 4.11 (2H, s, $H_{\alpha Gly}$, $H_{\alpha' Gly}$); 4.51 (1H, br, $H_{\alpha Leu}$); 5.15 (1H, d, $J^3NH_{Leu} - H_{\alpha Leu} = 8.41$ Hz).

Synthesis of Boc-Leu-Δ^ZPhe Oxazolone (4)

To a solution of 5 mmol of **3** in 15 ml of DCM, 5 mmol (500 µl) of benzaldehyde and 3.6 mmol (500 µl) of TEA were added. The mixture was stirred at room temperature overnight under an inert atmosphere. The solvent was removed under vacuum. **4** was obtained in a 90% yield as a yellow oil which was used without further purification.

IC-MS (NH4): 359 (100, M+1); 376 (17, M+18). ^{1}H-NMR ($CDCl_3$) δ ppm: 0.97 (2 × $3H_{\delta Leu}$, d, $J^3H_{\delta Leu} - H_{\gamma Leu} = 6.23$ Hz); 1.3 ($H_{\beta Leu}$, br); 1.44 (3 × $3H_{Boc}$, s); 1.6–1.8 (2H, br, $H_{\beta' Leu}$, $H_{\gamma Leu}$); 4.74 (1H, br, $H_{\alpha Leu}$); 4.98 (1H, d, NH_{Leu}, $J^3NH_{Leu} - H_{\alpha Leu} = 8.73$ Hz); 7.17 (1H, s, $H_{\beta \Delta ZPhe}$); 7.42 (3H, br, $H_{\varepsilon Aro}$, $H_{\varepsilon' Aro}$, $H_{\zeta Aro}$); 8.07 (2H, br, $H_{\delta Aro}$, $H_{\delta' Aro}$).

Synthesis of Boc-Leu-Δ^ZPhe-Gly-OMe (5)

To a solution of 565 mg (4.5 mmol) of H-GlyOMe.HCl in 10 ml of DCM, 626 µl (1 eq) of TEA was added to neutralize the solution, followed by 4.5 mmol of **4** in 15 ml of DCM and a catalytic amount of DMAP. The mixture was stirred overnight at room temperature, under an inert atmosphere. After removing the solvent, the resulting oil was dissolved in 50 ml of AcOEt, filtered, and washed with water, saturated aqueous $NaHCO_3$, saturated aqueous NaCl and then water to neutral pH. The organic phase was concentrated and purified on a silica gel column with DCM–acetone (80 : 20). **5** was obtained in an 87% yield.

IC-MS (NH4): 448 (21, M+1); 479 (100, M+18). ^{1}H-NMR ($CDCl_3$) δ ppm: 0.899 ($3H_{\delta Leu}$, d, $J^3H_{\delta Leu} - H_{\gamma Leu} = 5.80$ Hz); 0.953 ($3H_{\delta' Leu}$, d, $J^3H_{\delta' Leu} - H_{\gamma Leu} = 5.86$ Hz); 1.4 (3 × $3H_{Boc}$, s); 1.45–1.54 ($H_{\gamma Leu}$, m br); 1.57–1.67 (2H, m br, $H_{\beta Leu}$, $H_{\beta' Leu}$); 3.71 ($3H_{OMe}$, s); 3.93 (1H, dd, $H_{\alpha Gly}$, $J^3H_{NHGly} - H_{\alpha Gly} = 5.09$ Hz, $J^2H_{\alpha' Gly} - H_{\alpha Gly} = 17.68$ Hz); 4.14 (2H, m, $H_{\alpha Leu}$, $H_{\alpha' Gly}$, $J^3H_{NHGly} - H_{\alpha' Gly} = 5.87$ Hz, $J^2H_{\alpha' Gly} - H_{\alpha Gly} = 17.61$ Hz); 5.24 (1H, d, NH_{Leu}, $J^3NH_{Leu} - H_{\alpha Leu} = 5.38$ Hz); 7.32 (3H, m br, $H_{\varepsilon Aro}$, $H_{\varepsilon' Aro}$, $H_{\zeta Aro}$); 7.43 (3H, m br, $H_{\delta Aro}$, $H_{\delta' Aro}$, $H_{\beta \Delta ZPhe}$); 7.56 (1H, large s, $NH_{\Delta ZPhe}$); 8.10 (1H, s, NH_{Gly}).

Synthesis of Boc-Leu-MeΔ^ZPhe-Gly-OMe (6)

To a solution of 874 mg (1.95 mmol) of **5** in 20 ml of DMF, 121 µl (2 mmol) of CH_3I and 1.1 g (8 mmol) of K_2CO_3 were added. The mixture was stirred at room temperature under an inert atmosphere and away from the light for 72 h. The solvent was removed under vacuum. The resulting oil was dissolved in AcOEt, filtered and washed with saturated aqueous NaCl and then water to neutral pH. The organic phase was concentrated and purified on a silica gel column with AcOEt–heptane (3 : 1). **6** was isolated in quantitative yield.

IC-MS (NH4): 462 (100, M+1); 479 (9, M+18). ^{1}H-NMR ($CDCl_3$) δ ppm: 0.569 ($3H_{\delta Leu}$, d, $J^3H_{\delta Leu} - H_{\gamma Leu} = 6.72$ Hz); 0.610 ($3H_{\delta' Leu}$, d, $J^3H_{\delta' Leu} - H_{\gamma Leu} = 6.72$ Hz); 0.896 (1H, m br, $H_{\beta Leu}$); 1.170 (1H, m br, $H_{\gamma Leu}$); 1.278 (1H, m br, $H_{\beta' Leu}$); 1.406 (3 × $3H_{Boc}$, s); 3.238 (3H, s, $NCH3_{\Delta ZPhe}$); 3.747 (3H, s, OMe); 4.156 (3H, m br, $H_{\alpha Leu}$, $H_{\alpha Gly}$, $H_{\alpha' Gly}$); 4.788 (1H, d, NH_{Leu}, $J^3NH_{Leu} - H_{\alpha Leu} = 6.09$ Hz); 7.434 (5H, s, $H_{Aro \Delta ZPhe}$); 7.747 (1H, s, $H_{\beta \Delta ZPhe}$); 8.386 (1H, large s, NH_{Gly}).

Synthesis of Boc-MeAla-Leu-MeΔ^ZPhe-Gly-OMe (9a)

To a solution of 0.47 mmol of **6**, deprotected on the *N*-terminal side by the general protocol of *N*-Boc deprotection, in 25 ml of MeCN, 216 mg (0.564 mmol, 1.2 eq) of HBTU and 200 µl (3 eq) of TEA were added. A solution of 0.47 mmol of Boc-MeAla-OH (**7a**) in 1 ml of MeCN was added. The mixture was stirred for 30 min at room temperature. The solvent was removed and the product was purified on a silica gel column with DCM–acetone (70 : 30). The product **9a** was isolated in a 92% yield.

IC-MS (NH4): 547 (100, M+1); 564 (85, M+18). ^{1}H-NMR ($CDCl_3$) δ ppm: 0.557 ($3H_{\delta Leu}$, d, $J^3H_{\delta Leu} - H_{\gamma Leu} = 6.51$ Hz); 0.567 ($3H_{\delta' Leu}$, d, $J^3H_{\delta' Leu} - H_{\gamma Leu} = 6.32$ Hz); 1.209–1.388 (3H, m br, $H_{\beta Leu}$, $H_{\beta' Leu}$, $H_{\gamma Leu}$); 1.255 (3H, d, $H_{\beta Ala}$, $J^3H_{\alpha Ala} - H_{\beta Ala} = 6.97$ Hz); 1.441 (3 × $3H_{Boc}$, s); 2.666 (3H, s, $NCH3_{Ala}$); 3.209 (3H, s, $NCH3_{\Delta ZPhe}$); 3.709 (3H, s, OMe); 4.130 (2H, 2d, $H_{\alpha' Gly}$, $H_{\alpha Ala}$, $J^3H_{\alpha Ala} - H_{\beta Ala} = 6.25$ Hz); 4.326

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(1H, dd, $H_{\alpha Gly}$, $J^2H_{\alpha Gly} - H_{\alpha' Gly} = 10.95$ Hz), 4.639 (1H, m br, $H_{\alpha Leu}$); 7.354 (1H, s, NH_{Leu}); 7.407 (5H, s, $H_{Aro\Delta ZPhe}$); 7.720 (1H, s, $H_{\beta\Delta ZPhe}$); 8.509 (1H, large s, NH_{Gly}).

Synthesis of Boc-$^{14}CH_3$Ala-Leu-MeΔ^ZPhe-Gly-OMe (9b)

As described for **9a**, 7 mg (15 μmol) of **6** (*N*-Boc deprotected) was reacted with 27.2 MBq (735 μCi) (13.6 μmol) of Boc-$^{14}CH_3$Ala-OH (**7b**) in MeCN in the presence of HBTU (1.2 eq) and TEA (4 eq) to give 24.5 MBq (663 μCi) of **9b**, 90% radioactive yield from **7b**; specific activity: 2 GBq/mmol (54 mCi/mmol). IC-MS (NH4): 549 (100, M + 1). The ^{1}H-NMR spectrum of **9b** was identical to that of **9a**.

Synthesis of Boc-MeAla-Leu-Δ^ZPhe-Gly-OMe (8)

245 mg (0.55 mmol) of **5**, deprotected on the *N*-terminal side using the general protocol of *N*-Boc deprotection, was reacted with 1 eq of **7a**, using the method described for the synthesis of **9a**, to give **8** in a 90% yield.

IC-MS (CH4): 533 (100, M+1). ^{1}H-NMR ($CDCl_3$) δ ppm: 0.887 ($3H_{\delta Leu}$, d, $J^3H_{\delta Leu} - H_{\gamma Leu} = 6.10$ Hz); 0.949 ($3H_{\delta' Leu}$, d, $J^3H_{\delta' Leu} - H_{\gamma Leu} = 6.30$ Hz); 1.316 (3H, d, $H_{\beta Ala}$, $J^3H_{\alpha Ala} - H_{\beta Ala} = 7.13$ Hz); 1.406 ($3 \times 3H_{Boc}$, s); 1.574–1.688 (2H, m br, $H_{\beta Leu}$, $H_{\gamma Leu}$); 1.720–1.805 (1H, m br, $H_{\beta' Leu}$); 2.792 (3H, s, $NCH3_{Ala}$); 3.741 (3H, s, OMe); 4.107 (2H, m, $H_{\alpha Gly}$, $H_{\alpha' Gly}$); 4.301 (1H, m br, $H_{\alpha Leu}$); 4.470 (1H, q, $H_{\alpha Ala}$, $J^3H_{\alpha Ala} - H_{\beta Ala} = 7.10$ Hz); 6.707 (1H, large s, NH_{Leu}); 7.210–7.365 (4H, m br, $NH_{\Delta ZPhe}$, $H_{\varepsilon\Delta ZPhe}$, $H_{\varepsilon'\Delta ZPhe}$, $H_{\zeta\Delta ZPhe}$); 7.394–7.413 (3H, m br, $H_{\beta\Delta Zphe}$, $H_{\delta\Delta ZPhe}$, $H_{\delta'\Delta ZPhe}$); 7.945 (1H, large s, NH_{Gly}).

Synthesis of Boc-MeAla-Leu-$^{14}CH_3\Delta^Z$Phe-Gly-OMe (9c)

To a solution of 250 mg (0.47 mmol) of **8** in 20 ml of anhydrous DMF, 550 mg of K_2CO_3 (4 eq) and 814 MBq (22 mCi) (0.42 mmol) of $^{14}CH_3I$ were added by vacuum transfer (specific activity: 1.92 GBq/mmol (52 mCi/mmol). The mixture was isolated away from light and stirred at room temperature for 24 h. The solvent was removed under vacuum. The resulting oil was dissolved in AcOEt, filtered and washed with saturated aqueous NaCl and water to neutral pH. The organic phase was concentrated and purified on a silica gel column with AcOEt–acetone–hexane (2 : 1 : 1). 651 MBq (17.6mCi) of **9c** was obtained, 80% radioactive yield from $^{14}CH_3I$. Specific activity: 1.92 GBq/mmol (52 mCi/mmol) IC-MS (NH4): 549 (100, M + 1). The ^{1}H-NMR spectrum of **9c** was identical to that of **9a**.

Synthesis of Cyclo-(MeAla-Leu-MeΔ^ZPhe-Gly): Tentoxin (1a)

To a solution of 0.280 mmol of **9a**, with successively deprotected terminal acid and amine functions, using the general protocols of O-Me deprotection and *N*-Boc deprotection, in 60 ml of anhydrous DMF, 234 μl (6 eq) of TEA was added. A solution of 118 mg of HATU (0.308 mmol, 1.1 eq) in 1 ml of anhydrous DMF was added. The mixture was stirred for 30 min at room temperature. The solvent was removed and the product was purified on a silica gel column with AcOEt–MeOH (80 : 10). The cyclotetrapeptide **1a** was isolated in an 80% yield. This compound was eluted at 27.038 min in analytical RP-HPLC. IC-MS (CH4): 415 (100, M + 1). ^{1}H-NMR ($CDCl_3$) δ ppm: 0.485 ($3H_{\delta Leu}$, d, $J^3H_{\delta Leu} - H_{\gamma Leu} = 5.54$ Hz); 0.605 ($3H_{\delta' Leu}$, d, $J^3H_{\delta' Leu} - H_{\gamma Leu} = 6.78$ Hz); 1.217–1.320 (2H, m br, $H_{\beta Leu}$, $H_{\gamma Leu}$); 1.530 (3H, d, $H_{\beta Ala}$, $J^3H_{\alpha Ala} - H_{\beta Ala} = 7.19$ Hz); 1.583–1.661 (1H, m br, $H_{\beta' Leu}$); 2.786 (3H, s, $NCH3_{Ala}$); 3.173 (3H, s, $NCH3_{\Delta ZPhe}$); 3.558 (1H, d, $H_{\alpha outGly}$, $J^2H_{\alpha inGly} - H_{\alpha outGly} = 15.07$ Hz); 4.190 (1H, large m br, $H_{\alpha Leu}$); 4.361 (1H, m br, $H_{\alpha Ala}$, $J^3H_{\alpha Ala} - H_{\beta Ala} = 6.45$ Hz); 5.179 (1H, dd, $H_{\alpha inGly}$, $J^2H_{\alpha inGly} - H_{\alpha outGly} = 14.76$ Hz, $J^3NH_{Gly} - H_{\alpha inGly} = 10.71$ Hz), 7.289 (1H, s, NH_{Leu}); 7.390 (5H, s, $H_{Aro\Delta ZPhe}$); 7.723 (1H, s, $H_{\beta\Delta ZPhe}$); 8.159 (1H, large s, NH_{Gly}).

Synthesis of Cyclo-($^{14}CH_3$Ala-Leu-MeΔ^ZPhe-Gly): $^{14}CH_3$Ala-tentoxin (1b)

As described for **1a**, 15.65 MBq (423 μCi) **1b** was obtained from 19.54 MBq (528 μCi) (9.8 μmol) of **9b**, 80% radioactive yield. Specific activity of **1b**: 2 GBq/mmol (54 mCi/mmol). IC-MS (CH4): 417 (100, M + 1). The ^{1}H-NMR spectrum of **1b** was identical to that of **1a**. This compound was eluted at 27.480 min in analytical RP-HPLC.

Synthesis of Cyclo(MeAla-Leu-$^{14}CH_3\Delta^Z$Phe-Gly): $^{14}CH_3\Delta^Z$Phe-tentoxin (1c)

As described for **1a**, 120 MBq (3.25 mCi) of **1b** was obtained from 148 MBq (4 mCi) (77 μmol)

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of **9c**, 81% radioactive yield. Specific activity of **1b**: 1.92 GBq/mmol (52 mCi/mmol). IC-MS (CH4): 417 (100, M + 1). The ^{1}H-NMR spectrum of **1c** was identical to that of **1a**. This compound was eluted at 26.811 min in analytical RP-HPLC.

GENERAL PROCEDURE FOR CYCLIZATION

Protocol A: Cyclization with DPPA, HBTU or HATU

To a solution of **9c**, with successively deprotected terminal acid and amine functions, using the general protocols of O-Me deprotection and *N*-Boc deprotection, dissolved in anhydrous DMF or anhydrous acetonitrile, TEA was added. This solution was stirred at the temperature indicated in Tables 2 and 3, and the coupling reagent (DPPA, HBTU or HATU) in solution in DMF was then added in one go. The ratios of the reagents are indicated in Tables 2 and 3. These studies were conducted with radioactivity levels of **9c** between 192 KBq (5.2 μCi) and 59.2 MBq (1600 μCi), corresponding respectively to 0.1 μmol and 30.8 μmol of the linear tetrapeptide. The reaction was complete after 1 h. The yields were measured by radiochromatography on silica TLC, mobile phase: AcOEt–MeOH (90 : 10). R_f tentoxin: 0.59.

Protocol B: Cyclization with DCC/PfpOH

To a solution of 2 μmol (3.85 MBq, 104 μCi) of **9c**, with deprotected terminal acid function, dissolved in 0.8 ml of freshly distilled THF, 3 eq of PfpOH and 1.5 eq of DCC were added, with stirring. The mixture was stirred at room temperature for 18 h and the solvent was then removed under vacuum. The resulting crude product was dissolved in 1 ml of DCM, and cooled to 0 °C to precipitate and filter DCU. To the filtered solution 0.5 ml of TFA was added. The mixture was stirred at room temperature for 1 h. The solvent was removed and the resulting crude product was then dissolved in 1 ml of DCM and evaporated (twice). The solid residue was dissolved in a mixture of 5 ml of dioxane and 1 ml of pyridine. This mixture was stirred at room temperature under an inert atmosphere for 24 h. The yields were measured by radiochromatography on silica TLC, mobile phase: AcOEt–MeOH (90 : 10). R_f tentoxin: 0.59.

Table 3 Study of the Cyclization Conditions with HATU and HBTU for the Synthesis of $^{14}CH_3\Delta^{Z}$Phe-tentoxin

Entry[a]	1 mM	Reagent (eq)	Base (eq)	Solvent	Yield[b] (%)
1	0.3	HATU (3.0)	TEA (3)	DMF	61
2	0.3	HATU (3.0)	TEA (10)	DMF	78
3	0.3	HATU (1.1)	TEA (3)	MeCN	27
4	0.3	HATU (1.1)	TEA (10)	MeCN	74
5	1.1	HATU (3.0)	TEA (10)	DMF	79
6	10.2	HATU (3.0)	TEA (10)	DMF	78
7	1.0	HBTU (1.1)	TEA (3)	DMF	73
8	1.0	HBTU (1.1)	TEA (6)	DMF	74

[a] General method of cyclization: the tetrapeptide was dissolved in the solvent with TEA, the cyclization reagent HBTU or HATU was introduced rapidly in one addition.
[b] The yields were measured by radiochromatography on silica TLC, mobile phase: AcOEt/MeOH (90 : 10). R_f tentoxin: 0.59.

Table 2 Comparative Study of Coupling Reagents for the Synthesis of $^{14}CH_3\Delta^{Z}$Phe-tentoxin

Entry[a]	1 mM	Reagent (eq)	Base (eq)	Temp.	Solvent	Yield[c] (%)
1	0.01	DPPA (3)	TEA (3)	0 °C	DMF	29
2[b]	0.33	DCC/PfpOH	Pyridine	rt	Pyridine/dioxane	60
3	1	HBTU (1.1)	TEA (3)	rt	DMF	73
4	0.5	HATU (1.1)	TEA (6)	rt	DMF	81

[a] General method of cyclization: the tetrapeptide was dissolved in the solvent with TEA, the cyclization reagent DPPA, HBTU or HATU was introduced rapidly in one addition.
[b] The protocol of Rich *et al.*[10, 11] was used for the cyclization.
[c] The yields were measured by radiochromatography on silica TLC, mobile phase: AcOEt/MeOH (90 : 10). R_f tentoxin: 0.59.

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RESULTS AND DISCUSSION

Synthesis of H-R[1]Ala-Leu-R[2]Δ^ZPhe-Gly-OH (12)

The main difficulty of the linear tetrapeptide synthesis lies in the introduction of the dehydro amino acid.

Δ^ZPhe (or α–acetamido cinnamic acid) is commercially available. The vinyl group deactivates the acid and amine functions, so the coupling yields with this amino acid are very low. To solve this problem, Rich and Mathiaparanam [10,11] added benzyl mercaptan on the vinyl function non-stereoselectively, and then synthesized the linear tetrapeptide Boc-MeAla–Leu-Phe(3S-Bzl)-Gly-OMe in the solid phase. Oxidation with sodium metaperiodate, followed by dehydrosulfenylation gave the Z and E isomers of Boc-MeAla-Leu-ΔPhe-Gly-OMe. After *N*-methylation of the dehydro residue, the Z isomer was isolated in a 19% yield from the α-acetamido cinnamic acid.

Jacquier and Verducci [12] activated the α-acetamido cinnamic acid using an oxazolone before coupling it successively with leucine and glycine. The yield remained low and the linear tetrapeptide Boc-MeAla-Leu-MeΔ^ZPhe-Gly-OMe was obtained in a 43% overall yield from the α-acetamido cinnamic acid.

Edwards *et al.* [13] obtained the Z dehydro residue by stereoselective dehydration of the Boc-Leu-D,L-phenylserine azlactone. This synthetic route afforded Boc-MeAla-Leu-MeΔ^ZPhe-Gly-OMe in a 45% overall yield from D,L-3-phenylserine.

Cavelier and Verducci [14] used the same strategy to prepare the dehydro residue. However, the azlactone of Boc-Leu-phenylserine was formed *in situ* by oxidation to DDQ of the Boc-Leu-Phe azlactone. A 47% overall yield of Boc-MeAla-Leu-MeΔ^ZPhe-Gly-OMe was obtained from Boc-Leu-Phe-OH. This method offers the advantage of using a naturally occurring starting material.

Our method (Scheme 1) consists of synthesizing the dehydro-oxazolone by reaction of an aldehyde with an oxazolone according to a modification of the azlactone synthesis of Erlenmeyer [18–21]. The oxazolone of Boc-Leu-Gly (**3**), prepared by reaction of DCC with Boc-Leu-Gly-OH (**2**), generates the azlactone of Boc-Leu-phenylserine *in situ* by an aldolization reaction with benzaldehyde in the presence of TEA. The oxazolone of Boc-Leu-phenylserine was dehydrated spontaneously and stereoselectively to the oxazolone of Boc-LeuΔ^ZPhe (**4**), which was obtained in a 90% yield. Although, the Erlenmeyer azlactone synthesis is not stereoselective, (E)-azlactones are thermolabile and isomerize to their corresponding (Z)-isomers in the presence of tertiary amine [19–21]. The glycine methyl ester was coupled with the oxazolone **4** in the presence of TEA and a catalytic amount of DMAP. The tripeptide Boc-Leu-Δ^ZPhe-Gly-OMe (**5**) was obtained in an 87% yield. All three steps can be carried out in one pot with a similar 78% total yield from Boc-Leu-Gly-OH.

The methylation of the tripeptide **5** was achieved stereoselectively on the Δ^ZPhe by 1.025 eq of methyl iodide and an excess of potassium carbonate. The tripeptide Boc-Leu-MeΔ^ZPhe-Gly-OMe (**6**) was isolated in quantitative yield. After deprotection of its terminal amine function, this peptide **6** was coupled with Boc-MeAla-OH (**7a**) in the presence of HBTU. The tetrapeptide Boc-MeAla-Leu-MeΔ^ZPhe-Gly-OMe (**9a**) was isolated in a 92% yield. In the same way, the radiolabelled tetrapeptide, Boc-$^{14}CH_3$Ala-Leu-MeΔ^ZPhe-Gly-OMe (**9b**), was synthesized starting from Boc-$^{14}CH_3$Ala-OH (**7b**) in a 90% yield.

This method, easy to implement from naturally occurring, commercially available and inexpensive materials, introduces the dehydro amino acid stereospecifically with a good yield. The total yield of tetrapeptide **9** from dipeptide **2** is higher than 70%. Also, the aldolization-crotonization reaction of azlactones can be carried out with other aldehydes [20], which should make possible the syntheses of new tentoxin analogues modified on the dehydro residue.

It is generally best to label molecules at the latest possible synthesis step. To label the tentoxin on the *N*-methyl of Δ^ZPhe, the Boc-MeAla-Leu-Δ^ZPhe-Gly-OMe (**8**) was first synthesized by coupling **5** with **7a** in a 92% yield and then methylating with (^{14}C)-methyl iodide in an 80% yield. Boc-MeAla-Leu-$^{14}CH_3\Delta^Z$Phe-Gly-OMe (**9c**) was obtained in a 58% total yield from Boc-Leu-Gly-OH.

Cyclization

Small peptides, in particular tetrapeptides, are generally difficult to cyclize and require to be worked in a highly dilute solution. The yields are usually low. A great number of cyclization reagents have been studied. However, it is difficult to compare them because the cyclization yield of a peptide varies according to the number, nature and sequence of the amino acids that compose it [22,23].

In 1982, Schmidt [24] advocated the use of pentafluorophenol activated ester as a cyclization reagent for small peptides. In 1996, Ehrlich [25] showed, on pentapeptide models, a high capacity for the cyclization of esters activated by

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reagents derived from 1-hydroxy-7-azabenzotriazole (HOAt). The cyclization reagents used in the four syntheses of tentoxin were: DCC–2,4,5-trichlorophenol [10,11], DPPA [14] and the DPPA–DMAP–HOBt mixture [13,26], respectively giving 18%, 19% and 52% yields. The best results obtained by Edwards with a 52% cyclization yield suggest that HOBt improves the yield. This study shows that three activated esters are likely to give good results: pentafluorophenol, HOBt and HOAt esters. Thus we selected three reagents (DCC–PfpOH, HBTU and HATU [27,28]) to give these activated esters and compared them with DPPA.

For this study, we used the linear (^{14}C)-tetrapeptide **9c**. The radiolabelling makes it possible to measure the cyclization yield easily and accurately.

The optimized results of this study are summarized in Table 2. DPPA gave a low 29% yield in **1c** (Entry 1). DCC–PfpOH (Entry 2) and HBTU (Entry 3) gave good yields, respectively 60% and 73%. HATU gave an excellent yield of 81% (Entry 4), confirming that the derivatives of HOAt are more effective than those of HOBt [25,27]. These last conditions used to carry out cyclization afforded tentoxin and its labelled analogues in an 80% yield of the isolated product.

However, the cyclization yield obtained with HATU ranged widely according to the quantity of TEA used (Table 3). This was particularly surprising as these results were independent of the concentration of the linear tetrapeptide. An increase of the concentration of TEA caused an increase in the cyclization yield from 61% (Entry 1) to 78% (Entry 2) in DMF and from 27% (Entry 3) to 74% (Entry 4) in a less polar solvent such as MeCN. On the other hand, an increase in the concentration of the starting peptide from 0.3 to 10.2 mM did not cause any considerable reduction in the cyclization yield, which remained about 78% (Entries 2, 5 and 6).

The increase in the concentration of TEA did not cause any notable modification of cyclization yield with HBTU (Entries 7 and 8). The influence of the concentration in TEA was not related to a characteristic of the linear peptide but to a specificity of HATU. On the other hand, the cyclization capacity of an activated ester did not depend on the concentration of TEA but only on the ratio of its inter and intra rate constants which defines the effective molarity EM [29, 30] (Note 1). In this context it is probable that the concentration of TEA interferes during the formation of the activated ester. In spite of the close structural homology between HATU and HBTU, the mechanism of the activated ester formation, usually accepted for HBTU [31], cannot be applied to HATU. The very weak variation in the yield for concentrations of the linear tetrapeptide from 0.3 to 10 mM implies that EM is greater than 10^{-2}M. This indicates a surprising cyclization capacity of the activated OAt ester. In our present state of knowledge, it is not possible to attribute this facility of cyclization to any specific conformation of the linear peptide or to the activating group.

According to Carpino [27], the difference in the coupling efficiency of the activating groups OAt and OBt could arise from the chelation of one proton of the terminal amine function of the peptide by the nitrogen of the aromatic ring of the OAt group. This principle of basic catalysis was suggested by Jakubke [32] to explain the enhanced reactivity of esters of 8-hydroxyquinoline. This type of chelation applies to the intramolecular reaction that affords the activated ester in the pseudo-cyclic form (Figure 2). Obviously such a conformation should facilitate the cyclization.

The ^{1}H-NMR parameters of compounds **1a**, **1b** and **1c** (Table 1 and Figure 3) were in conformity

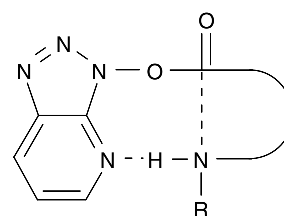

Figure 2 Pseudo-cyclic form of activated ester.

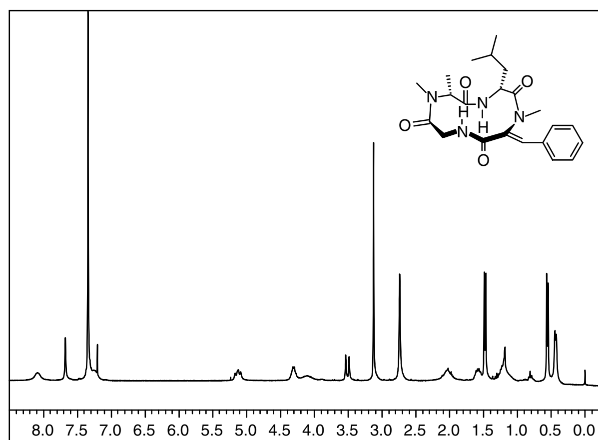

Figure 3 ^{1}H NMR spectrum (300 MHz) of tentoxin **1a** taken in chloroform-d.

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with those obtained by Rich [16]. Analytical RP-HPLC showed coelution of **1a**, **1b** or **1c** with natural tentoxin, isolated and purified from *Alternaria alternara* strain i 1248/28.

NOTE

1. Effective molarity (EM) is the concentration of the cyclizable species, the activated ester of the tetrapeptide, for which the intramolecular reaction rate is equal to the intermolecular reaction rate. The order of the intermolecular reaction is 1 and that of the intermolecular reaction is 2. EM is thus equal to the ratio of the rate constants: $EM = k_{intra}/k_{inter}$.

CONCLUSION

The various total syntheses of tentoxin published to date gave low total yields (max. 20%). Two difficulties explain these poor results: the introduction of the dehydro residue and the cyclization. A new method for the introduction of the dehydro residue and the use of HATU as a cyclization reagent afforded a marked increase in the total yield (60%). This synthetic route appears sufficiently adaptable and effective to be used for the synthesis of new analogues of tentoxin needed for the structure/function study of chloroplastic ATPase and P450-3A.

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