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Trans-4-aminoproline, a phytotoxic metabolite with herbicidal activity produced by *Ascochyta caulina*

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Abstract

A phytotoxic metabolite, characterized through NMR techniques and synthetic methods as *trans*-4-aminoproline, was isolated from the culture filtrates of *Ascochyta caulina*, a promising mycoherbicide for biological control of *Chenopodium album*. The metabolite, which shows interesting phytotoxic properties, together with ascaulitoxin (recently characterized as N^2-β-D-glucoside of the unusual bis-amino acid 2,4,7-triamino-5-hydroxyoctandioc acid) and another unidentified compound, compose an active fraction of *A. caulina* culture filtrates with promising herbicidal properties. When assayed on leaves of host and non host dicots, including wild and cultivated plants, the *trans*-4-aminoproline showed a wide range of toxicity, with leaves of *C. album* being the most sensitive. Other interesting aspects were its inefficacy on several monocots, both cultivated and wild, and its lack of antifungal, antibiotic and zootoxic activities. This is the first report on *trans*-4-aminoproline as naturally occurring compound and phytotoxic metabolite produced by *A. caulina*.

Keywords: *Chenopodium album*; *Ascochyta caulina*; Phytotoxins; Nonproteigenic amino acids; *Trans*-4-aminoproline; Weed biocontrol

1. Introduction

The perthotrophic fungal species *Ascochyta caulina* (P. Karst.) v.d. Aa and v. Kest. has been proposed as a mycoherbicide against *Chenopodium album* (L.) (Kempenaar, 1995). This is known as common lambsquarter or fat hen and is a common world-wide weed of many arable crops as sugar beet and maize (Holm, Pluckett, Pancho & Herberger, 1977). The application of pycnidiospores of the fungus to *C. album* plants causes the appearance of large necrosis of leaves and stems, and depending on the amount of necrosis developed, plants show retarded growth or death. Preliminary experiments had showed that *A. caulina* produced in vitro phytotoxic hydrophilic low molecular weight metabolites (Scheepens, Kempenaar, Adreanas, Eggers, Netland & Vurro, 1997), which could be relevant for an alternative, or in addition, to the use of pathogens in weed biocontrol (Strobel, Kienfield, Bunkers, Sugawara & Clardy, 1991). Recently, the main phytotoxin, named ascaulitoxin, has been isolated and characterized as N^2-β-D-glucoside of the unusual bis-amino acid 2,4,7-triamino-5-hydroxyoctandioc acid (Evidente et al., 1998). Considering that the other toxic metabolites also exhibited an amino acidic nature, the culture filtrates were preliminarily fractionated by cationic-exchange chromatography obtaining a mixture of phytotoxins. The purification of this mixture by gel filtration chromatography allowed isolation of a further phytotoxin which, together with ascaulitoxin and other still unknown metabolites, is responsible for the high phytotoxicity of the fungal culture filtrate.

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Table 1
^{1}H- and ^{13}C-NMR D_2O data of *trans*-4-aminoproline (**1**) and *trans*-4-hydroxy-L-proline (**3**)

C	**1**[a]					HMBC	**3**				
	δ	*m*	δH	*m*	*J* (Hz)		δ	*m*	δH	*m*	*J*
2	58.4	*d*	4.71	*dd*	9.8, 8.4		55.6	*d*	4.35	*dd*	10.2, 7.9
3	28.1	*t*	2.82	*ddd*	12.3, 9.8, 9.8	4.71, 4.55, 3.83, 3.79	40.1	*t*	2.43	*brdd*	14.1, 7.9
			2.44	*ddd*	12.3, 9.8, 8.4				2.17	*ddd*	14.1,10.2,4.4
4	60.0	*d*	4.55	*m*		2.82, 2.44	72.7	*d*	4.67	*m*	
5	63.6	*t*	3.83	*dd*	13.0, 3.7		62.5	*t*	3.49	*dd*	12.7, 3.8, 1.8
			3.79	*dd*	13.0, 5.1				3.37	*brd*	12.7, 1.48
COOH	177.1	*s*				4.71, 2.82, 2.44	176.9	*s*	–	–	–

[a] 2D ^{1}H, ^{1}H (COSY) and 2D ^{13}C, ^{1}H (HMQC) NMR experiments delineated correlations of all protons and corresponding carbons.

This paper reports on the isolation and chemical and biological characterization of this new phytotoxin.

2. Results and discussion

The culture filtrate of *A.caulina*, showing high phytototoxicity on leaves and cuttings of both host and non-host plants, was fractionated by cation-exchange chromatography, which allowed separation of the mixture of phytotoxic metabolites from other metabolites and the large amount of saccharose used as carbon source in the culture medium. The mixture of toxic metabolites was successively purified by a Bio-Gel P-2 column, as detailed in Section 3, yielding the second fraction group containing ascaulitoxin (Evidente et al., 1998) and the third one corresponding to a homogeneous compound characterized as *trans*-4-aminoproline (**1**, 150 mg l^{-1}), as described below.

Assayed at 1 μg/μl on punctured leaves, the toxin had a drastic effect on the host, causing the fast appearance of large necroses (6–7 mm diameter) surrounding the puncture. At concentration five times lower, the toxin was still active, causing smaller necroses. On other dicot leaves the phytotoxicity varied ranging from very wide necrosis (poppy, annual mercury, cucumber, wild cucumber), to medium (tree of Heaven, tomato, common sowthistle), to small (black nightshade). An interesting aspect is the lack of toxicity when **1** was assayed on several monocots, both cultivated (wheat, oat, barley) as well as wild (canarygrass, slender foxtail, wild oat). Tested up to 10^{-5} M on cut young fat-hen seedlings the toxin caused wide necrosis and dryness of cotyledons, while no effects could be seen on stems. The same symptoms on leaves but not on stems, could be observed on cut host plants, only at higher concentrations (10^{-4} M).

The toxin (**1**) proved to be a non-aromatic amino acid considering the typical reactivity of its TLC with ninhydrin associated to the lack of the characteristic UV absorption. It showed a molecular weight of 130 as deduced by mass spectrometry. In fact, the ions generated from the molecular ion by loss of H_2O and by cluster formation with both K^+ and Na^+ were observed in the FAB and ES mass spectra at *m*/*z* 112 and 169 and 153, respectively. The ^{1}H-NMR spectrum (see Table 1) showed signal systems very similar to those present in the spectrum of the proteigenic *trans*-4-hydroxy-L-proline (**3**) recorded in the same conditions. In particular, the double doublet of the α-

Table 2
^{1}H-NMR data of N^{1},N^{4}-ditosyl methyl ester derivatives of *trans*-4-aminoproline and *cis*-4-amino-L-proline (**2** and **9**)

H	**2**			**9**		
	δ	*m*	*J* (Hz)	δ	*m*	*J* (Hz)
2	4.39	*dd*	9.5, 7.6	4.21	*dd*	10.1, 2.3
3	2.44	*ddd*	11.8, 9.5, 6.4	2.14	*ddd*	14.6, 10.1, 6.30
3′	2.26	*ddd*	11.8, 7.6, 5.6	1.82	*br ddd*	14.6, 2.3, 2.3, 1.2
4	4.23	*m*		3.99	*m*	
5	4.21	*dd*	10.8, 4.6	3.25	*dd*	10.3, 4.9
5′	4.18	*dd*	10.8, 5.3	3.18	*ddd*	10.3, 2.4, 1.2
OMe	3.67	*s*		3.76	*s*	
Ph	7.80–7.35	*m*		7.72–7.32	*m*	
Me–Ph	2.47	*s*		2.45	*s*	
Me–Ph	2.46	*s*		2.43	*s*	

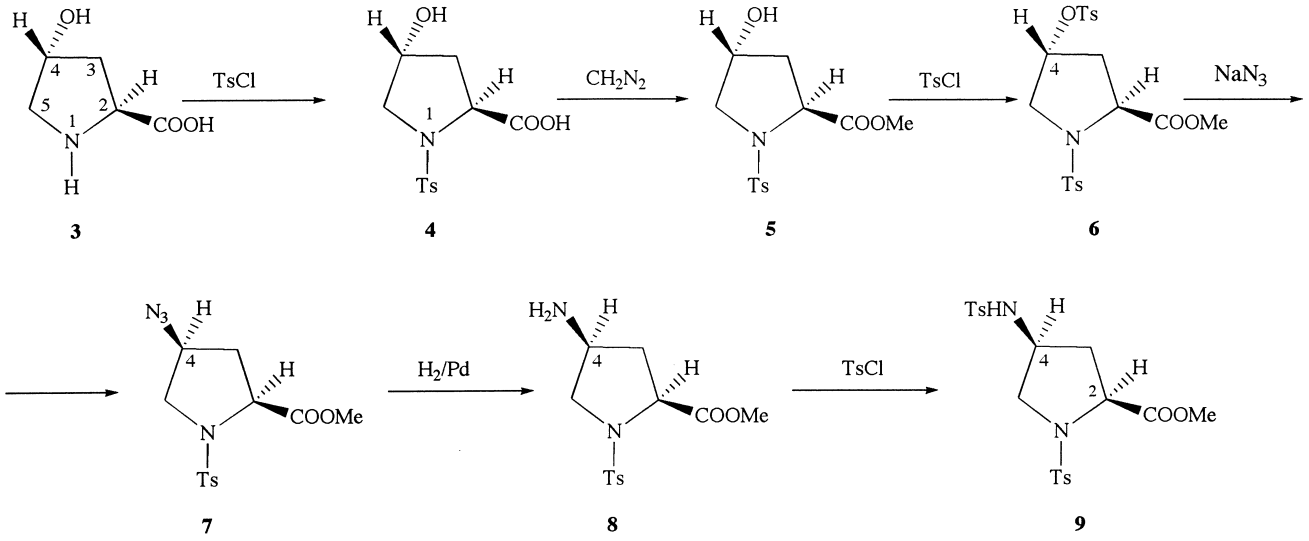


Fig. 1. Synthesis of N^1,N^4-ditosyl-*cis*-4-amino-L-proline methyl ester.

amino acidic proton (H-2) at δ 4.71, coupled, in the COSY spectrum (Bax & Freeman, 1981), with both protons of the adjacent methylene group (H_2C-3), which appeared as two double doublets at δ 2.82 and 2.44. Both of them correlated with the H-4 proton resonating as a complex multiplet at δ 4.55, a chemical shift characteristic of a secondary carbon bearing an amino group (Pretsch, Clerc, Seibl & Simon, 1983).

Finally, the latter coupled with the protons of a further methylene group (H_2C-5), appearing as two double doublets at δ 3.83 and 3.79, which are typical of the methylene bonded to the nitrogen of proline derivatives (Pretsch et al., 1983). Taken together, these data suggest the structure of 4-aminoproline for this new toxin. The ^{13}C-NMR data were consistent with those of **3** (Table 1) but with an amino group and a carboxylic groups at C-4 and C-2, respectively resonating at the typical chemical shift values of δ 60.0 and 58.4, together with the significant singlet of the carboxylic group observed at δ 177.1 (Pretsch et al., 1983; Breitmaier & Voelter, 1987). Furthermore, the structure **1** was corroborated by the correlations recorded in the HMQC spectrum (Table 1) (Bax, Ikura, Kay, Torchia & Tschudin, 1990).

The structure of 4-aminoproline was confirmed by preparing the corresponding N^1,N^4-ditosylmethyl ester derivative (**2**). Its ^{1}H-NMR (see Table 2), besides the signals of the two tosyl (Ts) groups and the singlet of the ester methoxy group at δ 3.67, showed a signal pattern very similar to that of **1** except for the downfield shift ($\Delta\delta$ 0.38 and 0.39, respectively) of both H-5 and H-5′, observed as two double doublets at δ 4.21 and 4.18. The EIMS did not show the molecular ion at *m*/*z* 452 but the ion at *m*/*z* 394, generated from it by loss of the α-lactone (2-etanolide), which is a fragmentation mechanism typical of proline derivatives (Porter, 1985). Similarly, the parent ion, by successive loss of CO_2Me and Ts residues, generated the ion at *m*/*z* 222. By an alternative fragmentation mechanism, the molecular ion, losing in succession two TsNH, or $TsNH_2$ residues, produced the ions at *m*/*z* 282, 92 and 281, respectively. The base peak, corresponding to the expected tropylium ion, was recorded at *m*/*z* 91.

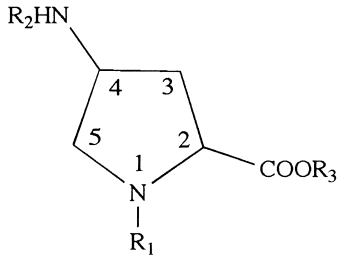
Considering that 4-aminoproline can exist as four stereoisomers and that biological activity of pyrrolidine derivatives depend on their substitution pattern, functionalization and absolute configuration (Massiot & Delaude, 1986; Numata & Ibuka, 1987; Tanaka, Suzuki & Sawanishi, 1996; Thorbek, Hjeds & Schaumburg, 1981), a study was undertaken to determine the stereochemistry of the toxin. Unfortunately, **1** and crystals of derivative **2** proved to be unsuitable for X-ray analysis. Therefore, the determination of the relative stereochemistry of 4-aminoproline was carried out by chemical methods and comparing the physical and the spectroscopic properties of the N^1,N^4-ditosyl methyl ester derivative, obtained from **1**, and from the commercially available *trans*-4-hydroxy-L-proline (**3**), whose absolute configuration is 2*S*, 4*R*.

The well known synthetic procedure (Andreatta, Nair, Robertson, & Simpson, 1967; Portoghese & Mikhail, 1966) adopted to convert **3** into the corresponding N^1,N^4-ditosyl methyl ester derivative, is depicted in Fig. 1. The proteigenic amino acid (**3**) was first transformed into the *N*-tosyl derivative (**4**), which in turn was esterified with ethereal diazomethane and then the resulting methyl ester (**5**) converted into the corresponding *N*,*O*-ditosyl derivative (**6**). The latter,

by nucleophilic substitution, was transformed into the corresponding azido derivative (**7**) with inversion of the configuration at C-4. This was first reduced by catalytic hydrogenation and the resulting very unstable amino derivative converted into N^1,N^4-ditosyl-*cis*-4-amino-L-proline methyl ester (**9**). Derivative **9** proved to be different from the N^1,N^4-ditosyl methyl ester (**2**) prepared starting from **1**. In fact, **2** and **9** showed different optical rotations and TLC behaviours in three different systems (see Section 3). Moreover, their ^1H-NMR spectra (Table 2), recorded in the same conditions, appeared significantly different in terms of chemical shifts and coupling constants. In particular, comparing **2** and **9**, the H-3′, H-4 and both H-5 and H-5′ appeared lowfield shifted ($\Delta\delta$ 0.44, 0.24, 0.96 and 1.00, respectively) at δ 2.26, 4.23, 4.21 and 4.18, respectively, while the coupling constants between H-3′ with both H-2 and H-4 and H-4 with H-5′ were markedly different. On these bases the two ditosyl methyl ester derivatives (**2** and **9**) appeared to be diastereomers and therefore the relative stereostructure of *trans*-4-aminopyrrolidine-2-carboxylic acid can be assigned to 4-aminoproline (**1**). Furthermore, the specific optical rotation value ($[\alpha]_D^{21}$ +47.2) measured for **1** and that reported for the *trans*-4-amino-L-proline ($[\alpha]_D^{21}$ −57.8) shared their enantiomeric relation, therefore suggesting the absolute *trans*-4-amino-D-proline stereostructure for **1**. Further attempts are in progress to confirm this relative and possibly absolute stereochemistry by X-ray analysis of a suitable derivative.

Chiral non racemic pyrrolydines are common structural subunits found in many natural and synthetic products with biological activity (Massiot & Delaude, 1986; Numata & Ibuka, 1987). Biological activities of pyrrolidines are affected by both the functionalization and stereochemistry of the pyrrolidine ring (Massiot & Delaude, 1986; Numata & Ibuka, 1987; Tanaka et al., 1996; Thorbek et al., 1981). Among these, cucurbitine (Tanaka et al., 1996; Fang, Li, Niu & Tseng, 1961; Shiao, Shao, Ho, Yang & Mao, 1962), containing (*S*)-α-amino acid function at 3-position in the pyrrolidine ring and *cis*-3-amino-L-proline (Hatanaka, 1969) are the naturally occurring non proteigenic bioactive amino acids more related to *trans*-4-aminoproline (**1**). *Trans*-4-aminoproline is already known as synthetic product (Andreatta et al., 1967; Mauger & Witkop, 1966), but this is its first report as naturally occurring compound and as phytotoxin produced by *A. caulina* with potential bioherbicidal activity.

Considering the high phytotoxic activity of the compound on fat-hen, the range of activity for other dicots, and its inefficacy against monocots associated to the lack of antifungal, antibiotic and zootoxic activity in the used assays, investigations on the practical use of the toxin as natural herbicide, as alternative or in addition to the use of the producing pathogen are very interesting and are in progress.



1 R_1=R_2=R_3=H

2 R_1=R_2=Ts, R_3=Me

3. Experimental

3.1. General

Mp were taken on a Reichert Thermovar and are uncorrected; optical rotations were measured in $CHCl_3$ on a JASCO DIP 370 Digital polarimeter, unless otherwise noted; IR spectra were recorded (neat) on a Perkin-Elmer FT-IR 1720X spectrometer. UV spectra were taken on a Perkin-Elmer Lamba 3B UV/Vis spectrophotometer in MeCN, unless otherwise noted. ^{1}H- and ^{13}C-NMR: were recorded in $CDCl_3$ (unless otherwise noted) at 500 or 250 and at 125 or 62.5 MHz, respectively, on DRX 500 and AM 250 Bruker spectrometers. The same solvent used as internal standard. For spectra recorded in D_2O, TPS (sodium-3-trimethylsylilpropionate-2,2,3,3-d_4) was used as internal standard. Carbon multiplicities were determined by DEPT spectra (Breitmaier & Voelter, 1987). DEPT, COSY-45, HMQC and HMBC (Bax & Summers, 1986) NMR experiments were performed using Bruker microprograms. EI and Electrospray MS were recorded at 70 eV on a Fisons Trio-2000 and a Perkin-Elmer API-100 spectrometer, respectively. FAB MS were recorded in glycerol/thioglycerol using Cs as bombarding atoms on a VG ZAB 2SE. Analytical and preparative TLC were performed on silica gel (Merck, Kieselgel, 60 F_{254}, 0.25 and 0.50 mm, respectively) or on reverse phase (Whatman, KC18 F_{254}, 0.20 mm) plates; the spots werc visualized by exposure to UV radiation and/or by spraying with 0.5% ninhydrin in Me_2CO followed by heating at 110°C for 10 min. CC: Dowex-50 and Bio-Gel P-2. Solvent systems: (A) BuOH–HOAc–H_2O (3:1:1); (B) *iso*-PrOH–H_2O (7:3); (C) $CHCl_3$-*iso*-PrOH (49:1); (D) $CHCl_3$-*iso*-PrOH (97:3), (E) $CHCl_3$-*iso*-PrOH (9:1), (F) $CHCl_3$-*iso*-PrOH (19:1); (G) EtOAc-*n*-hexane (1:1); (H) EtOH–H_2O (1.5:1).

3.2. Production and bioassays of trans-4-aminoproline (1)

A strain of *A. caulina* (P. Karst) v.d. Aa and v. Kest freshly isolated from diseased leaf of *C. album* was kindly supplied by Dr P.C. Scheepens, AB-DLO, Wageningen, The Netherlands, and stored as single spore culture in the Collection of Istituto Tossine e Micotossine da Parassiti Vegetali, CNNR, Bari, Italy (ITEM 1058). The method used for the preparation of culture filtrates of *A. caulina* is that presented in a recent paper (Evidente et al., 1998). Culture filtrates, chromatographic frs and *trans*-4-aminoproline were assayed on host plants using the leaf-puncture assay. The pure toxin was assayed, using the same assay, at 1 μg/μl also on monocots, both cultivated (oat: *Avena sativa*, barley: *Hordeum vulgare*; durum wheat: *Triticum durum*) and weedy (canarygrass: *Phalaris canariensis*; slender foxtail: *Alopecurus myosuroides*; wild oat: *Avena fatua*), as well as on dicots, cultivated (tomato: *Lycopersicon esculentum*; cucumber: *Cucumis sativus*) or weeds (poppy: *Papaver rhoeas*; annual mercury: *Mercurialis annua*; wild cucumber: *Ecballium elaterium*, black nightshade: *Solanum nigrum*; tree of Heaven: *Ailanthus glandulosa*; common sowthistle: *Sonchus oleraceus*)

Trans-4-aminoproline was also assayed on young cut fat-hen seedlings (having only cotyledons) up to 5×10^{-6} M as well as on cut host plants (at the 4-true-leaf-stage, up to 8×10^{-5} M. Plants were cut and immersed in the toxin solution (or in water as control) for 2 days under continuous light, then were transferred in water. Symptoms were observed daily up to 5 days.

The antifungal activity of the toxins was checked on *Geotrichum candidum*, while the antibiotic one was assayed on *Pseudomonas syringae* subsp. *syringae* and on *Escherichia coli*, as already described in detail, up to 50 μg/disk (Evidente et al., 1998). The zootoxic activity was tested on *Artemia salina* brine shrimps up to 40 μg/ml of sea solution (Bottalico, Logrieco & Visconti, 1989).

3.3. Purification and characterization of trans-4-aminoproline (1)

The lyophilized culture filtrate of *A. caulina* (10 g, corresponding to 275 ml) was dissolved in 1 N HCOOH (3 ml) and adsorbed on a Dowex-50 H^+ form resin packed in a chromatographic column (2×30 cm). The column was washed with ultrapure water (50 ml), which permitted to remove both the great amount of saccharose used as carbon source in the culture medium, and other not basic substances. Than, the column was eluted with 1 N NH_4OH (300 ml) and the eluate was fluxed by a N_2 stream to remove ammonia. The residual lyophilized solution gave a residue consisting of a mixture of phytotoxic amino acids (243 mg). An aliquot of this (11.0 mg) was purified by a Bio-Gel P-2 column (3×57 cm) equilibrated and eluted with ultrapure Milli-Q water, as in detail previously described (Evidente et al., 1998); five groups of homogeneous fractions were obtained. Only residues of groups 2 (2.9 mg), 3 (1.8 mg) and 5 (3.9 mg) showed phytotoxic activity. The residue obtained from group 2 resulted to be ascaulitoxin (Evidente et al., 1998), while that of group 3 proved to be another homogeneous compound (150 mg/l of culture filtrate) as showed by TLC analysis on silica gel and reverse phase (R_f 0.21 and 0.75, using eluents A and B, respectively). It was identified as *trans*-4-aminoproline (**1**): $[\alpha]_D^{21}$: +47.2 (*c* 0.4, H_2O) UV (H_2O) λ_{max} nm (log ε) <220; ^{1}H- and ^{13}C-NMR: Table 1; FAB (+) *m/z* (rel. int.): 112 $[M\text{-}H_2O]^+$ (100); ES *m/z*: 169 $[M + K]^+$, 153 $[M + Na]^+$.

3.4. N^1,N^4-Ditosyl-trans-4-aminoproline methyl ester (2)

The *trans*-4-aminoproline(**1**, 11, 0 mg) was dissolved in 2 N NaOH (2 ml) and shaken for 1 day with a TsCl Et_2O solution (75 mg/2 ml). The aqueous layer was separated, acidified with 6 N HCl and refrigerated overnight. The yielded crude product (15.2 mg) was separated by filtration and its cold solution in MeOH (2 ml) was treated with a ethereal CH_2N_2, until a pale yellow color persisted. The solvent was removed in vacuo and the white residue purified by prep. TLC (silica gel, eluent C) to give **2** as a homogeneous compound [15.0 mg; R_f 0.69 and 0.47 (silica gel eluent F and G, respectively) and 0.41 (reverse phase, eluent H)]: $[\alpha]_D^{25}$: +19.8 (*c* 0.3); UV λ_{max}, nm (log ε) 225 (4.3); IR ν_{max} cm^{-1}: 1731, 1597; ^{1}H-NMR: Table 2; EIMS, *m/z* (rel. int.): 394 $[M\text{-}COOCH_2$ (α-lactone)$]^+$ (9), 282 $[M\text{-}TsNH]^+$ (6), 281 $[M\text{-}TsNH_2]^+$ (44), 268 $[M\text{-}CHO\text{-}Ts]^+$ (72), 240 $[M\text{-}HCO\text{-}CO\text{-}Ts]^+$ (21), 222 $[M\text{-}CO_2Me\text{-}Ts]^+$ (77), 155 $[Ts]^+$ (98), 92 $[M\text{-}2 \times TsNH]^+$ (33), 91 $[C_7H_7]^+$ (100). ES: *m/z*, 491 $[M+K]^+$, 475 $[M+Na]^+$, 453 $[M+H]^+$.

3.5. Synthesis of N^1,N^4-ditosyl-cis-4-amino-L-proline methyl ester (9)

Trans-4-hydroxy-L-proline (**3**, 500 mg) was converted, according to described procedures (Andreatta et al., 1967; Portoghese & Mikhail, 1966) and as depicted in Fig. 1, into the corresponding *N*-tosyl derivative (**4**, 500 mg). The latter was esterified with CH_2N_2 and the resulting methyl ester (**5**, 450 mg) converted into the corresponding *N*,*O*-ditosyl derivative (**6**, 400 mg), which in turn, by nucleophilic substitution with NaN_3 was transformed in the expected *N*-tosyl-

cis-4-azido-L-proline (**7**, 200 mg). The intermediates **4**–**7** were characterized by the following physical and spectroscopic properties compared with the very few spectroscopic data available from literature (Andreatta et al., 1967; Portoghese & Mikhail, 1966).

3.6. N-Tosyl-trans-4-hydroxy-L-proline (*4*)

4 had: mp 148–150°C; $[\alpha]_D^{25}$: −101.3 (*c* 0.6, EtOH); [Portoghese & Mikhail, 1966: mp 153–155°C.; $[\alpha]_D^{23}$ −105.4° (*c* 2% EtOH)]; UV (MeOH) λ_{max} nm (log ε) 228 (4.0); IR ν_{max} cm^{-1}: 3395, 1736, 1598; ^{1}H-NMR data (CD_3OD), δ: 7.77 (2H, Ph), 7.41 (2H, Ph), 4.35 (1H, *m*, H-4), 4.25 (1H, *t*, $J_{2,3} = J_{2,3'} = 7.9$ Hz, H-2), 3.59 (1H, *dd*, $J_{4,5} = 4.2$ and $J_{5,5'} = 10.9$ Hz, H-5), 3.28 (1H, *ddd*, $J_{3,5'} = 1.5$, $J_{4,5'} = 2.6$ and $J_{5,5'} = 10.9$ Hz, H-5′), 2.44 (3H, *s*, MePh), 2.11 (2H, *m*, H_2-3); EI MS, *m*/*z* (rel. int.): 240 $[M\text{-}COOH]^+$ (43), 155 $[Ts]^+$ (29), 91 $[C_7H_7]^+$ (81), 86 $[M\text{-}Co_2\text{-}Ts]^+$ (30), 58 (100)

3.7. N-Tosyl-trans-4-hydroxy-L-proline methyl ester (*5*)

5 had: mp 98–100°C; $[\alpha]_D^{25}$ −102.1 (*c* 0.5); [Andreatta et al., 1967: mp 103–104°C; $[\alpha]_D^{20}$ −98.54° (*c* 1% $CHCl_3$)]; UV λ_{max} (log ε) 225 (4.3); IR ν_{max} cm^{-1}: 3515, 1741, 1598; ^{1}H-NMR: δ: 7.79 (2H, Ph), 7.32 (2H, Ph), 4.46 (1H, *m*, H-4), 4.43 (1H, *t*, $J_{2,3} = J_{2,3'} = 8.1$ Hz, H-2), 3.75 (3H, *s*, OMe), 3.62 (1H, *dd*, $J_{4,5} = 4.1$ and $J_{5,5} = 11.4$ Hz, H-5), 3.40 (1H, *dt*, $J_{4,5'} = J_{3,5'} = 1.8$ and $J_{5,5'} = 11.4$ Hz, H-5′), 2.44 (3H, *s*, MePh), 2.20 (1H, *m*, H-3), 2.13 (1H, *m*, H-3′); EIMS, *m*/*z* (rel. int.): 240 $[M\text{-}CO_2Me]^+$ (49), 155 $[Ts]^+$ (40), 91 $[C_7H_7]^+$ (100), 58 (55).

3.8. N,O-Ditosyl-trans-4-hydroxy-L-proline methyl ester (*6*)

6 had: mp 92–94°C; $[\alpha]_D^{25}$: −49.5 (*c* 0.5); [Andreatta et al., 1967: mp 95°C; $[\alpha]_D^{20}$ −54.8° (*c* 2% $CHCl_3$)]; UV λ_{max} nm (log ε) 225 (4.3); IR ν_{max} cm^{-1}: 1757, 1598; ^{1}H-NMR, δ: 7.72 (2H, Ph), 7.63 (2H, Ph), 7.34 (2H, Ph), 7.29 (2H, Ph), 5.00 (1H, *m*, H-4), 4.29 (1H, *t*, $J_{2,3} = J_{2,3'} = 7.8$ Hz, H-2), 3.75 (3H, *s*, OMe), 3.69 (1H, *dd*, $J_{4,5} = 4.4$ and $J_{5,5'} = 12.3$Hz, H-5), 3.58 (1H, *dt*, $J_{3,5'} = J_{4,5'} = 1.9$ and $J_{5,5'} = 12.3$ Hz, H-5′), 2.47 (3H, *s*, MePh), 2.45 (3H, MePh), 2.35 (1H, *br ddd*, $J_{2,3} = 7.8$, $J_{3,3'} = 12.3$ and $J_{3,5'} = 1.9$ Hz, H-3), 2.20 (1H, *ddd*, $J_{2,3'} = 7.8$, $J_{3,3'} = 12.3$ and $J_{3',4} = 5.0$ Hz, H-3′); EIMS, *m*/*z* (rel. int.): 394 $[M\text{-}CO_2Me]^+$ (27), 240 $[M\text{-}CO_2Me\text{-}Ts]^+$ (50), 222 $[M\text{-}CO_2Me\text{-}Ts\text{-}H_2O]^+$ (98), 155 $[Ts]^+$ (96), 91 $[C_7H_7]^+$ (100).

3.9. N-Tosyl-cis-4-azido-L-proline methyl ester (*7*)

7 had: mp 65–68°C; $[\alpha]_D^{25}$: −44.3 (*c* 0.5); [Andreatta et al., 1967: mp 69–70°C; $[\alpha]_D^{20}$ −47.8° (*c* 2% $CHCl_3$)]; UV λ_{max} nm (log ε) 227 (4.1); IR ν_{max} cm^{-1}: 2108, 1757, 1598 [lit. 15: ν_{max} ($CHCl_3$) 2108, 1750]; ^{1}H-NMR, δ: 7.82 (2H, Ph), 7.34 (2H, Ph), 4.53 (1H, *dd*, $J_{2,3} = 9.0$ and $J_{2,3'} = 4.1$ Hz, H-2), 4.07 (1H, *m*, H-4), 3.75 (3H, *s*, OMe), 3.65 (1H, *dd*, $J_{4,5} = 6.0$ and $J_{5,5'} = 10.9$ Hz, H-5), 3.33 (1H, *ddd*, $J_{3,5'} = 1.0$, $J_{4,5'} = 4.0$ and $J_{5,5'} = 10.9$ Hz, H-5′), 2.46 (3H, *s*, MePh), 2.36 (1H, *dddd*, $J_{2,3} = 9.0$, $J_{3,3'} = 13.4$, $J_{3,4} = 4.5$ and $J_{3,5'} = 1.0$ Hz, H-3), 2.26 (1H, *dt*, $J_{2,3'} = J_{3'}$, $4 = 4.5$ and $J_{3,3'} = 13.4$ Hz, H-3′); EIMS, *m*/*z* (rel. int.): 265 $[M\text{-}CO_2Me]^+$ (55), 237 $[M\text{-}CO_2Me\text{-}N_2]^+$ (27), 155 $[Ts]^+$ (88), 91 $[C_7H_7]^+$ (100).

3.10. N^1,N^4-Ditosyl-cis-4-amino-L-proline methyl ester (*9*)

N-Tosyl-*cis*-4-azido-L-proline methyl ester (**7**, 24 mg) in MeOH (1 ml) was hydrogenated in the presence of 10% Pd on charcoal (100 μg) at room temperature and 1 atmospheric pressure. After 4 h the catalyst was filtrated off and the solvent was removed under vacuum. The reaction mixture (28 mg) was purified by TLC prep. on silica gel (eluent E) giving the very unstable *N*-tosyl-*cis*-4-amino-L-proline (**8**, 7.0 mg) as homogeneous compound, which was immediately converted into the corresponding ditosylderivative as follows: **8** (7 mg) was dissolved in dry pyridine (100 μl) and the solution was chilled in an ice-bath. A cold solution of TsCl (28 mg) in dry pyridine (100 μl) was added and the reaction mixture was left at 0° for 3 days. The oily residue left by the reaction work-up was purified by prep. TLC (silica gel, eluent F) giving N^1,N^4-ditosyl-*cis*-4-amino-L-proline methyl ester (**9**) as homogeneous compound (9.5 mg): $[\alpha]_D^{25}$: −10.4 (*c* 0.12); UV λ_{max} nm (log ε) 226 (4.3); IR ν_{max} cm^{-1}: 1738, 1598; ^{1}H-NMR: Table 2. EIMS, *m*/*z* (rel. int.): 394 $[M\text{-}COOCH_2$ (α-lactone)$]^+$ (2), 393 $[M\text{-}CO_2Me]^+$ (10), 282 $[M\text{-}TsNH]^+$ (4), 281 $[M\text{-}TsNH_2]^+$ (28), 268 $[M\text{-}CHO\text{-}Ts]^+$ (9), 222 $[M\text{-}CO_2Me\text{-}Ts]^+$ (100), 155 $[Ts]^+$ (81), 92 [M-2 × TsNH]+ (29), 91 $[C_7H_7]^+$ (86)

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