Pergamon

PII: S0031-9422(97)01072-8

*Phytochemistry*, Vol. 48, No. 7, pp. 1131–1137, 1998

Printed in Great Britain
0031-9422/98 $19.00+0.00

# ASCAULITOXIN, A PHYTOTOXIC *BIS*-AMINO ACID *N*-GLUCOSIDE FROM *ASCOCHYTA CAULINA*

ANTONIO EVIDENTE,* RENATO CAPASSO, ADELE CUTIGNANO, ORAZIO TAGLIALATELA-SCAFATI,† MAURIZIO VURRO,‡ MARIA CHIARA ZONNO‡ and ANDREA MOTTA§

Dipartimento di Scienze Chimico-Agrarie, Università di Napoli Federico II, Via Università 100, I-80055 Portici, Italy; † Dipartimento di Chimica delle Sostanze Naturali, Università di Napoli Federico II, Via D. Montesano 49, I-80131 Napoli, Italy; ‡ Istituto Tossine e Micotossine da Parassiti Vegetali, CNR, Viale L. Einaudi 51, I-70125 Bari, Italy: § Istituto per la Chimica di Molecole di Interesse Biologico,¶ CNR, Via Toiano 6, I-80072 Arco Felice, Italy

(*Received in revised form* 11 *November* 1997)

**Key Word Index**—*Chenopodium album*; Chenopodiaceae; *Ascochyta caulina*; mycoherbicide; phytotoxins; *N*-glucosides; nonproteigenic amino acids; ascaulitoxin.

**Abstract**—A new unusual phytotoxic *bis*-amino acid *N*-glucoside, named ascaulitoxin, was isolated from the culture filtrate of *Ascochyta caulina*, the causal agent of leaf and stem necrosis of *Chenopodium album*, a promising mycoherbicide for the biological control of this common noxious weed. Ascaulitoxin, characterized by extensive use of NMR techniques and chemical methods as $N^2$-(2,4,7-triamino-5-hydroxy)-octanedioyl-β-D-glucopyranoside, showed phytotoxic activity against host and non-host plants. 

## INTRODUCTION

Weeds have always been recognized as one of the most serious agricultural and environmental problems. In agriculture, the control of weed diffusion is usually achieved by using agrochemicals belonging to different classes of organic compounds, often in large amounts. This causes serious problems to human and animal health and produces heavy environmental pollution. On the contrary, biological agents offer the advantage of being fully compatible with the environment, often with high specificity, and represent a long-term solution also to control weeds, particularly those resistant to chemical herbicides. Therefore, many efforts have been made for weed biocontrol using their natural antagonists, mainly pathogens [1] and insects. Accordingly, the perthotrophic fungal species *Ascochyta caulina* (P. Karst.) v.d. Aa and v. Kest. has been proposed as a mycoherbicide against *Chenopodium album* [2], also known as common lambsquarter or fat hen, a common world-wide weed of arable crops, such as sugar beet and maize [3]. 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.

Considering that *A. caulina* belongs to a well-known toxin-producer genus [4], and the possible use of fungal toxins as an alternative or in addition to the use of pathogens in weed biocontrol [5], it seemed of interest to ascertain the production of toxic metabolites by *A. caulina*, and carry out their isolation and chemical and biological characterization.

## RESULTS AND DISCUSSION

The culture filtrate of *A. caulina*, showing high phytotoxicity on leaves and cuttings both of host and non-host plants, was examined to ascertain the chemical nature of the phytotoxic metabolites. They proved to be hydrophilic substances because they remained in the aqueous phase after exhaustive extraction carried out on the culture filtrates with organic solvents of increasing polarity (*n*-hexane < $CH_2Cl_2$ < EtOAc < BuOH). The phytotoxic metabolites had a $M_r$ lower than 1000, as deduced from dialysis experiments. These results prompted the purification of the crude culture filtrate by gel filtration. From a Biol-Gel P-2 column, eluted with ultrapure water, 14 groups of homogeneous fractions were obtained; only groups 4, 6 and 7 showed phytotoxic activity. The residue left from fraction group 4 proved to be the only homo-

* Author to whom correspondence should be addressed.

¶ Part of the 'Istituto Nazionale di Chimica dei Sistemi Biologici'.

geneous compound (235 mg $l^{-1}$), as shown by TLC analysis on silica gel and reverse-phase ($R_f$ 0.14 and 0.61, using eluents A and B, respectively). It was named ascaulitoxin (**1**).

Assayed on fat hen at 30 μg/droplet in the leaf-puncture assay, ascaulitoxin caused the appearance of necrotic spots surrounded by chlorosis. Particularly relevant in size was necrosis on sugarbeet (*Beta vulgaris*). Clear necrosis also appeared both on weeds [common sowthistle (*Sonchus oleraceous*), annual fleabane (*Erigeron annuus*), noogoora burr (*Xanthium occidentale*), Tree of Heaven (*Ailanthus glandulosa*)] and on cultivated plants [pea (*Pisum sativum*) and cucumber (*Cucumis sativus*)]. Still clear, but of reduced size, were necrosis on tomato (*Lycopersicon esculentum*) and redroot pigweed (*Amaranthus retroflexus*). The speed of symptom development varied between 2 and 5 days, depending on species.

When assayed on young fat hen cuttings at $8 \times 10^{-4}$ M, the toxin caused the necrosis of cotyledons, starting from their edges. On young tomato cuttings, ascaulitoxin caused a clear marginal necrosis of cotyledons and inhibited the production of adventitious roots, which well developed in controls. At $10^{-4}$ M the toxin caused 57 and 39% reduction of root elongation of fat hen and tomato seedlings, respectively. On the contrary, assayed up to 100 μg per disc on fungi (*Geotrichum candidum*) as well as on bacteria (*Pseudomonas syringae* and *Escherichia coli*), ascaulitoxin showed no antimicrobial activity.

Ascaulitoxin had a molecular formula of $C_{14}H_{27}N_3O_{10}$, corresponding to three degrees of unsaturation, as deduced from the $M_r$ of 397 measured by FAB mass spectrometry and from the HR-EI mass spectral data of its *penta*- and *hexa*-acetylderivatives. As the TLC chromatograms were visualised by spraying with ninhydrin, the presence of an amino acid moiety in **1** was suggested. This was confirmed by automatic amino acid analysis of ascaulitoxin, which was performed under standard conditions. The toxin showed basic behaviour, as it eluted with $R_t$ = 37.63 min, very similar to that of lysine ($R_t$ = 38.65 min). When the same analysis was performed on the residue left by the acid hydrolysed toxin, no peaks corresponding to standard proteigenic amino acids were observed. The TLC chromatograms of the acid hydrolysed product of **1**, obtained using the two systems indicated above and sprayed with the chromic acid mixture, also revealed a black spot typical of a glycidic residue. The presence of a glycidic and of a non-aromatic amino acid moiety in **1** was consistent with the absence of absorption maxima in the UV spectrum. 1D and 2D $^1H$ and $^{13}C$ NMR investigations indicated the presence of a β-glucopyranosyl residue and that of an atypical amino acid. In fact, the $^1H$ NMR spectrum (Table 1) showed a doublet ($J$ = 9.1 Hz) of an anomeric proton, which, compared to that of a β-*O*-glucopyranoside [6], appeared upfield at δ 4.04, a chemical shift value typical of β-*N*-glucopyranoside [7]. In the COSY-45 and TOCSY [8, 9] it correlated with proton systems typical of a β-glucopyranoside (Table 1) [6]. Furthermore, the $^1H$ NMR spectrum showed the system of the amino acid residue. A double doublet ($J$ = 10.0 and 7.5 Hz), typical of an α-amino-acid proton (H-2) [10] was observed at δ 4.32. In the COSY and TOCSY spectra, it correlated with two multiplets at δ 2.52 and 2.02, due to a methylene group ($H_2C$-3), which in turn coupled with the proton (H-4) of a secondary carbon, probably nitrogen-linked, present as a multiplet at δ 3.98 [10].

Table 1. $^1H$ and $^{13}C$ NMR data ($D_2O$) of ascaulitoxin (**1**)

| C* | δ | δH* | $J$ (Hz) | HMBC |
|---|---|---|---|---|
| 1 | 182.8 *s*† | | | 2.52, 2.02 |
| 2 | 56.0 *d* | 4.32 *dd* | 10.0, 7.5 | 4.04, 2.52, 2.02 |
| 3 | 24.0 *t* | 2.52 *m* | | 4.32, 3.98 |
| | | 2.02 *m* | | |
| 4 | 61.2 *d* | 3.98 *m* | | 2.52, 2.02, 1.67 |
| 5 | 73.0 *d* | 3.98 *m* | | 3.84, 2.02, 1.67 |
| 6 | 35.0 *t* | 2.04 *m* | | 3.84, 3.98 |
| | | 1.67 *m* | | |
| 7 | 57.2 *d* | 3.84 *dd* | 8.7, 5.0 | 2.04, 1.67 |
| 8 | 177.0 *s* | | | 3.84, 2.04, 1.67 |
| 1′ | 91.2 *d* | 4.04 *d* | 9.1 | 4.32, 3.96, 3.48, 3.37, 3.11 |
| 2′ | 72.8 *d* | 3.11 *dd* | 9.1, 9.1 | 4.04, 3.48 |
| 3′ | 80.0 *d* | 3.48 *dd* | 9.1, 9.1 | 3.37, 3.11 |
| 4′ | 72.5 *d* | 3.37 *dd* | 9.1, 9.1 | 3.96, 3.76, 3.48 |
| 5′ | 79.5 *d* | 3.40 *ddd* | 9.1, 5.2, 1.9 | 3.96, 3.76 |
| 6′A | 63.7 *t* | 3.96 *dd* | 12.2, 1.9 | 4.04, 3.40, 3.37 |
| 6′B | | 3.76 *dd* | 12.2, 5.2 | |

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

† Multiplicities determined by DEPT.

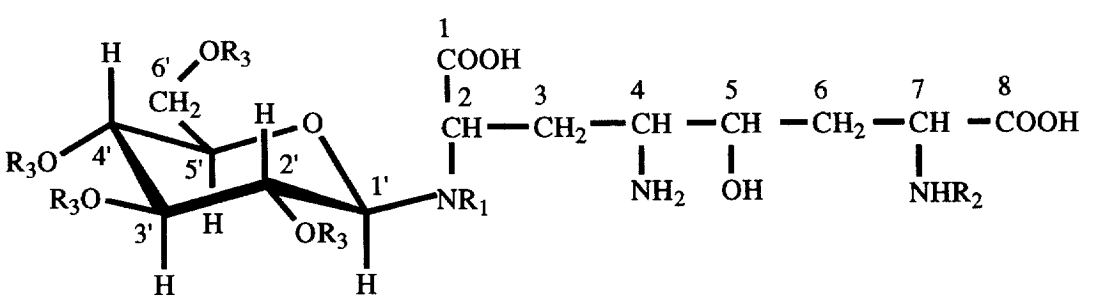
The latter overlapped with the multiplet of the proton (H-5) of another secondary carbon, which might be oxygenated, and coupled with the multiplets of another methylene group ($H_2C$-6) present at $\delta$ 2.04 and 1.67. Finally, the latter complex signals correlated with the double doublet ($J$ = 8.7 and 5.0 Hz) of H-7 at $\delta$ 3.84, which is a typical chemical shift value of an $\alpha$-amino-acid proton [10], as is that of H-2. Consequently, this moiety appears to be an unusual *bis*-amino acid, in which the two $\alpha$-carbons are connected by a 2,3-disubstituted butyl chain, with an $NH_2$ and hydroxyl groups.

The above results were consistent with the signal pattern observed in the $^{13}C$ NMR spectrum (Table 1). The singlets of the two carboxylic $\alpha$-amino-acid carbons appeared at $\delta$ 182.8 and 177.0 (C-1 and C-8, respectively), as well as the doublets of the two $\alpha$-amino acid carbons at $\delta$ 57.2 and 56.0 (C-7 and C-2) [11]. The two methylenes of the disubstituted butyl chain resonated at $\delta$ 35.0 and 24.0 (C-6 and C-3, respectively), while the other two secondary carbons appeared at $\delta$ 73.0 and 61.2. On the basis of their chemical shift values [11] and the correlations observed in the HMQC [12] spectrum, the latter were attributed to C-5 and C-4, which bear a hydroxyl and an amino group, respectively. The HMQC spectrum also confirmed the attributions of the other protons and the corresponding carbons of the amino acid residue, as well as those of the glucopyranosyl moiety. In particular, the assignment of the doublets at $\delta$ 4.04 to the anomeric proton, and $\delta$ 91.2 to the corresponding carbon, was further corroborated. The anomeric carbon (C-1′), compared to that of $\beta$-*O*-glucopyranoside [6, 11] and as already described for H-1′, appeared significantly upfield-shifted at a chemical shift value typical of $\beta$-*N*-glucopyranoside [7, 13]. The other carbons of the glucopyranosyl residue appeared with the expected multiplicities at typical $\delta$-values (Table 1) [6, 11]. In DMSO-$d_6$, the $^1H$ NMR spectrum of ascaulitoxin, in addition to the systems of the above partial structures, showed a broad signal for hydroxyl groups (at $\delta$ ~5.0) and two singlets at $\delta$ 7.56 and 7.15. As expected, the protons of the amino acid $\alpha$-$NH_2$ were not observed. The two singlets were attributed to the protons of the $NH_2$ located on C-4, one of which was probably engaged in a hydrogen bond with the hydroxyl group of the adjacent C-5 of the disubstituted butyl chain [10, 14], thus justifying their different spectroscopic behaviour. The hypothesised hydrogen bond appeared particularly stable as consequence of the 5-membered ring formation. The two partial structures found in **1** were confirmed by $^1H$ and $^{13}C$ NMR spectra recorded in DMSO-$d_6$ (data not shown). In particular, a significant long-range correlation was observed in the HMBC spectrum [15] between the nitrogen proton at C-4 at $\delta$ 7.15 and the $\alpha$-carbon (C-2) at $\delta$ 54.6.

On the basis of these results, the molecular formula of $C_{14}H_{27}N_3O_{10}$ and the molecular weight of 397, we hypothesised that ascaulitoxin is a glucopyranoside of the unusual 2,4,7-triamino-5-hydroxyoctanedioyl *bis*-amino acid.

The glycosylation site in **1** appeared to be the NH on the C-2, as deduced from the COSY and HMBC data of the toxin recorded in $D_2O$ (Table 1). In the COSY spectrum a significant long-range effect was observed between the anomeric proton (H-1′) at $\delta$ 4.04 and that of HC-2 at $\delta$ 4.32, while in the HMBC spectrum the anomeric carbon at $\delta$ 91.2 (C-1′) correlated with the protons H-2′, H-3′, H-4′ and H-6′A, but significantly also with H-2 at $\delta$ 4.32. In addition, in the same spectrum, the $\alpha$-carbon (C-2) at $\delta$ 56.0 was long-range coupled with the expected protons of the adjacent methylene ($H_2C$-3) at $\delta$ 2.52 and 2.02 but also with the anomeric proton at $\delta$ 4.04. The other correlations observed in the HMBC, as well as in the TOCSY and NOESY [16] experiments, confirmed the hypothesised structure as depicted in **1**.

The structure **1** assigned to ascaulitoxin was confirmed by chemical and spectroscopic methods. The FAB mass spectrum showed the presence of the $[M]^+$ at $m/z$ 398, which generated the intense ion at $m/z$ 380




**1** $R_1$=$R_2$=$R_3$=H

**2** $R_1$=$R_3$=Ac, $R_2$=H

**3** $R_1$=H, $R_2$=$R_3$=Ac

**4** $R_1$=$R_2$=$R_3$=Ac

Table 2. $^1$H NMR data ($CD_3OD$) of penta- and hexa-acetyl derivatives of ascaulitoxin (**2**–**4**)

| | **2***† | | **3***† | | **4**† | |
|---|---|---|---|---|---|---|
| H | $\delta$ | $J$ (Hz) | $\delta$ | $J$ (Hz) | $\delta$ | $J$ (Hz) |
| 2 | 4.66 *dd* | 10.2, 9.2 | 4.53 *dd* | 9.8, 9.6 | 4.63 *dd* | 7.0, 6.9 |
| 3 | 2.84 *ddd* | 13.1, 10.2, 2.9 | 2.84 *ddd* | 13.2, 9.8, 2.8 | 2.48 *m* | |
| | 2.26 *ddd* | 13.1, 9.2, 9.2 | 2.39 *ddd* | 13.2, 9.6, 9.6 | 2.15 *m* | |
| 4 | 4.70 *ddd* | 9.2, 3.2, 2.9 | 4.71 *ddd* | 9.6, 3.0, 2.8 | 4.78 *ddd* | 9.2, 3.0, 2.8 |
| 5 | 4.22 *ddd* | 9.4, 7.2, 3.2 | 4.27 *ddd* | 9.4, 7.1, 3.0 | 4.22 *ddd* | 9.4, 7.1, 3.0 |
| 6 | 2.51 *ddd* | 11.6, 9.4, 9.4 | 2.67 *ddd* | 12.2, 9.4, 9.4 | 2.32 *m* | |
| | 1.92 *ddd* | 11.6, 7.2, 7.2 | 2.21 *ddd* | 12.2, 7.1, 6.9 | 1.86 *m* | |
| 7 | 4.30 *dd* | 9.4, 7.2 | 4.73 *dd* | 9.4, 6.9 | 4.86 *m*‡ | |
| 1′ | 4.26 *d* | 9.4 | 4.17 *d* | 9.3 | 4.37 *d* | 9.2 |
| 2′ | 4.68 *dd* | 9.4, 9.4 | 4.87 *dd* | 9.3, 9.3 | 4.59 *dd* | 9.2, 9.2 |
| 3′ | 5.19 *dd* | 9.4, 9.4 | 5.21 *dd* | 9.3, 9.3 | 5.23 *dd* | 9.2, 9.2 |
| 4′ | 5.03 *dd* | 9.4, 9.4 | 5.09 *dd* | 9.3, 9.3 | 5.06 *dd* | 9.2, 9.2 |
| 5′ | 3.85 *ddd* | 9.4, 4.8, 2.3 | 3.87 *ddd* | 9.3, 4.9, 2.4 | 3.86 *ddd* | 9.2, 4.3, 2.8 |
| 6′A | 4.32 *dd* | 12.4, 4.8 | 4.33 *dd* | 12.4, 4.9 | 4.31 *dd* | 12.2, 4.3 |
| 6′B | 4.20 *dd* | 12.4, 2.3 | 4.19 *dd* | 12.4, 2.4 | 4.21 *dd* | 12.2, 2.8 |

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

† Acetyl groups appeared in the spectrum of **2**, **3** and **4** as singlets at $\delta$ 2.09, 2.04, 2.02, 2.00 and 1.93, at $\delta$ 2.09, 2.08, 2.02, 2.00 and 1.96 and at $\delta$ 2.09, 2.07, 2.02, 2.01, 2.00 and 1.94, respectively.

‡ This signal was, in part, overlapped with that of $H_2O$.

by losing $H_2O$. Methylalditol analysis of **1** gave the only significant peak in the GC and GC mass spectrum that corresponded to 1,5-diacetyl-2,3,4,6-tetramethyl glucose. It proved to be the D-stereomer from the results obtained from mass spectral analysis of the octanolyl hemiacetal, in turn obtained by reaction of the toxin with pure (+)-2-octanol, according to the method reported by Leontin *et al.* [17].

Ascaulitoxin was converted into the pentacetyl derivatives **2** and **3**, and the hexacetyl derivative **4**, the first being the main product of the reaction carried out with pyridine and acetic anhydride. Their $^1$H NMR spectra (Table 2) were very similar and all showed the expected downfield shifts of the geminal protons of the acetylable hydroxylated glucopyranosyl carbons H-2′, H-3′, H-4′ and H-6′A and H-6′B, but differed for the allocation of the remaining acetyl group(s). Considering that the presence of a stable hydrogen bond between the C-4 $NH_2$ and the C-5 hydroxyl group does not allow their derivatization, acylation is expected on the $\alpha$-amino group at C-2 or/and at C-7. This was confirmed by HR-EI and FAB mass spectral data which show the significant loss of $H_2O$, probably due to $\beta$-elimination of the free hydroxyl group at C-5 and the hydrogen of the adjacent $H_2C$-6 (see below).

Derivative **2** proved to be acetylated on the NH at C-2 because in its $^1$H NMR spectrum (Table 2) the adjacent proton (H-2) appeared downfield-shifted as a double doublet ($J$ = 10.2 and 9.2 Hz) at $\delta$ 4.66. This was supported by the long-range correlation observed in its HMBC spectrum between the methyl of the acetyl group at $\delta$ 21.0 and one of the two protons of the $H_2C$-3 at $\delta$ 2.26. As expected, the other pentacetyl derivative **3** showed acetylation of the $NH_2$ allocated at C-7, whose adjacent proton appeared significantly downfield-shifted in the corresponding $^1$H NMR spectrum as a double doublet ($J$ = 9.4 and 6.9 Hz) at $\delta$ 4.73. As expected, derivative **4** showed both protons (H-2 and H-7) downfield-shifted as a double doublet ($J$ = 7.0 and 6.9 Hz) and a multiplet at $\delta$ 4.63 and 4.86, respectively. In the spectra of all acetyl derivatives, the signals of the two methylenes ($H_2C$-3 and $H_2C$-6), as well as those of the HC-4 and HC-5, were similar in terms of chemical shift values and multiplicity. The EI and FAB mass spectra of the derivatives confirmed their structure and, therefore, that of **1**. The EI mass spectrum of **4** showed the $[M]^+$ at $m/z$ 649 and significant fragmentation peaks at $m/z$ 589, 529 and 469, produced by consecutive losses of three HOAc molecules. The $[M]^+$ by an alternative fragmentation mechanism, successively losing two $H_2O$ molecules, generated the ions at $m/z$ 631 and 613. The first one might be eliminated by formation of a stable $\delta$-lactam between the $NH_2$ group at C-4 and the carboxyl at C-7 (or alternatively by the formation of a $\gamma$-lactam between the same $NH_2$ group and the carboxyl at C-2). The second was probably lost through a $\beta$-elimination of the free hydroxyl group at C-5 and the hydrogen of the adjacent $H_2C$-6. It should be noted that the alternative loss of the second $H_2O$ molecule between the same hydroxyl group and the hydrogen at C-4 does not occur because it would generate an unstable enol-amino intermediate. Finally, the ion of the tetracetylglucosyl, appearing at $m/z$ 331, by loss of

two consecutive HOAc, $CH_2CO$ and HOAc generated the ions at *m/z* 271, 211, 169 and 109, respectively. In the HR-EI mass spectrum of **4**, the ion at 529.185326 corresponded to the expected formula of $C_{22}H_{31}N_3O_{12}$. Its FAB mass spectrum showed a $[MH]^+$ at *m/z* 650 and significant fragmentation peaks at *m/z* 590 and 530, due to the loss of two consecutive HOAc molecules. The same ion, losing alternatively $H_2O$ or $CO_2$, generated ions at *m/z* 632 or 606. The tetracetylglucosyl appeared significantly at *m/z* 331. The HR-EI mass spectrum of **2** did not show the $[M]^+$ but that at *m/z* 571.208969 ($C_{24}H_{33}N_3O_{13}$) produced from it by loss of two $H_2O$ molecules, as explained above for **4**. This ion generated by successive losses of $CO_2$, $CH_2CO$ and HOAc the ions at *m/z* 527, 485, and 425. Furthermore, by an alternative fragmentation pathway the ion at *m/z* 571, by successive losses of HOAc and $CH_3COO$, produced the ions at *m/z* 511 and 452, while the ion at *m/z* 527 losing in succession two HOAc molecules yielded the ions *m/z* 467 and 407. In addition, the tetracetylglucosyl appeared at *m/z* 331, together with the ions formed from its loss of HOAc and $CH_2CO$ residues, as reported above for **4**. Its FAB mass spectrum did not show a pseudomolecular ion but that formed, as described above, by loss of two $H_2O$ molecules, at *m/z* 572. This latter ion after loss of $CH_2CO$ generated the ion at *m/z* 530, while by alternatively losing a further $H_2O$ molecule, produced the ion at *m/z* 554. The HR-EI and FAB mass spectra of **3** showed ions at *m/z* 571.204861 ($C_{24}H_{33}N_3O_{13}$) $[M-2\times H_2O]^+$ and 572 $[MH-2\times H_2O]^+$, respectively, and fragmentation peaks, including those of the tetracetylglucosyl, very similar to those described above for **2**.

Therefore, ascaulitoxin may be formulated as $N^2$-(2,4,7-triamino-5-hydroxy)-octanedioyl-β-D-glucopyranoside. We intend to determine the stereochemistry at C-2, C-4, C-5 and C-7 by stereoselective synthesis of the toxin.

The structure and biological characterisation of ascaulitoxin have been described extensively herein. The finding of an *N*-glucoside of an atypical *bis*-amino acid is not surprising because nonproteigenic amino acids frequently occur as free or peptide components of animals, higher plants, algae and microorganisms, including fungi [18, 19]. Many of them have very unusual structures and interesting biological properties, such as antibiotic and fungicide activities [18, 19]. Considering its interesting phytotoxicity on *C. album*, and the lack of activity against fungi and bacteria, further studies are in progress on the role of ascaulitoxin in plant disease and on the mechanism of action. These aspects are important because the toxin could be used indirectly as biomarker for the improvement of *A. caulina* as mycoherbicide. If the toxin is a virulence factor, then more virulent strains of the pathogen could be more easily found, selecting the most toxigenic fungi. Moreover, these studies could permit the evaluation of possible direct use of the metabolite as a natural herbicide, either in combination with toxic metabolites present in the culture filtrate of *A. caulina* or with the pathogen itself, as well as with other control methods in the integrated weed management approach. Considering that the toxin alone does not seem to be able to penetrate into the leaf, applications to the leaf of the toxin in combination with the pathogen (which otherwise is very specific) could result in a selective treatment. The pathogen could be helped by the toxin, during the penetration and colonization stage to increase the disease level on the weed, without any effect on the non-host plant.

## EXPERIMENTAL

### *General*

Optical rotation and UV: in $H_2O$. $^1H$ and $^{13}C$ NMR: in $D_2O$ and/or DMSO-$d_6$ or $CD_3OD$, at 500, 400 and 300 or 125, 100 and 75.7 MHz, respectively, using the same solvent as int. standard. For spectra recorded in $D_2O$, TSP (sodium 3-trimethylsylil propionate-2,2,3,3-$d_4$) was used as int. standard. Carbon multiplicities were determined by DEPT spectra [11]. DEPT, COSY-45, TOCSY, HMQC, HMBC and NOESY NMR expts were performed using Bruker microprograms. EI and HR-EI MS: 70 eV. FAB MS: glycerol/thioglycerol using Cs as bombarding atoms. Amino acid analysis: hydrolysis 6 N HCl at 110° at 20 h. Analyser equipped with post column ninhydrin detection system. Analytical TLC: silica gel (Merck, Kieselgel, 60 $F_{254}$, 0.25 mm) or on reverse-phase (Whatman, KC18 $F_{254}$, 0.20 mm) plates; spots were visualised by spraying with a 5% ninhydrin $Me_2CO$ or first with 10% $H_2SO_4$ in MeOH and then with 5% phosphomolybdic acid in MeOH, or with chromic acid mixture, followed by heating at 110° for 10 min. CC: Bio-Gel P-2 or silica gel (Merck, Kieselgel, 60, 0.063–0.20 mm); solvent systems: (A) BuOH–HOAc–$H_2O$ (3:1:1); (B) *iso*-PrOH–$H_2O$ (7:3); (C) $CHCl_3$–MeOH (9:1). Dialysis expts were carried out using tubes with cut-offs of $M_r$ 12,000, 3500 and 1000.

*Production, purification and characterisation of ascaulitoxin* (**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, within the EC COST 816 project on "Biological control of weeds in Europe" and stored as single spore culture in the Collection of "Istituto Tossine e Micotossine da Parassiti Vegetali, CNR, Bari, Italy (ITEM 1058)". For production of toxic metabolites, the fungus was maintained on potato-dextrose-agar medium. Conidial suspension (1 ml containing approximately $10^6$ conidia) was added to 1 l Roux bottles containing 200 ml of M-1 D medium [20]. The cultures were incubated under static conditions at 25° in the dark for 4 weeks, then filtered, tested for phytotoxic activity and then lyophilised. An aliquot of the lyophilised material (2 g, corresponding to 55 ml of culture filtrate, which

contained an abundant amount of saccharose used as carbon source in the culture medium) was purified on a Bio-Gel P-2 column. The column (4.5 × 170 cm) was equilibrated and eluted with ultrapure Milli-Q water at flow rate of 1.3 ml $min^{-1}$ collecting a death vol. of 220 ml. Frs of 8 ml each were collected and monitored by UV absorption at 210 nm and by TLC on silica gel (eluent A) and reverse-phase (eluent B). On the basis of the chromatographic profiles and TLC evidence, the eluted frs were pooled into 14 groups of homogeneous frs, of which the groups 4 and 6–7 showed phytotoxic activity. In particular, by TLC analysis, groups 6 and 7 (33.3 and 75.8 mg, respectively) were shown to contain a mixt. of metabolites, while the residue (12.9 mg, 235 mg $l^{-1}$) from group 4 proved to be a homogeneous compound ($R_f$ 0.14 and 0.61 by TLC on silica gel and reverse-phase silica using eluents A and B, respectively), which therefore was named ascaulitoxin (**1**). $[\alpha]_D^{25}$ −26.5 (*c* 0.2). UV $\lambda_{max}$ nm (log ε): <210. $^1H$ and $^{13}C$ NMR: Table 1. FAB MS *m/z* (rel. int.): 398 $[MH]^+$ (25), 380 $[MH-H_2O]^+$ (100).

*Methylation, acid hydrolysis and acetylation of* **1**. To a soln of vacuum-dried ascaulitoxin (**1**, 0.6 mg) in $Me_2SO$ (500 μl) were added 2 M $KCH_2SOMe$ (125 μl) and MeI (60 μl) [21]. The crude reaction product was filtered through a C-18 Sep-Pack cartridge (Waters) previously washed with EtOH (20 ml), MeCN (2 ml) and $H_2O$ (10 ml). Frs were eluted with $H_2O$ (50 ml), $H_2O$–MeCN (4:1) (8 ml), MeCN (2 ml) and EtOH (4 ml). The last two frs were pooled and evapd to give the methylated *N*-glucoside, which was hydrolysed with 2 N TFA at 120° for 1 h. The partially methylated product in the hydrolysate was reduced with $NaBD_4$, acetylated [1:1 pyridine–$Ac_2O$ (60 μl) at 120° for 20 min] and analysed by GC-MS on a SP-2330 capillary column (Supelco, 30 m × 0.25 mm i.d., flow rate 0.8 ml $min^{-1}$, He as carrier gas), with a temp. prog.: 80° for 2 min, up to 170° at 30° $min^{-1}$, 170° for 0 min, up to 240° at 4° $min^{-1}$, 240° for 10 min. GC of methylated alditol acetates was carried out on a column identical with that used for GC-MS (flow rate 1 ml $min^{-1}$, $N_2$ as carrier gas), with the same temp. prog. using carbon response factors [22], and normalising the peak areas with respect to that of *myo*-inositol hexa-acetate used as int. standard.

*Determination of absolute stereochemistry of glucosyl residue of* **1**. Ascaulitoxin (**1**, 0.5 mg) was hydrolysed with 2 N TFA at 120° for 1 h, then few drops of *iso*-PrOH were added and the soln dried in a stream of $N_2$. To the residue was added a drop of TFA and optically pure (+)-2-octanol (0.5 ml), and the reaction mixt. left at 130° with stirring. After 18 h, the mixt. was concd in a stream of $N_2$ at 55°, dried under vacuum and then acetylated with pyridine (30 μl) and $Ac_2O$ (30 μl) at 100° for 20 min. Excess reagent was eliminated by co-distillation with EtOH. The same procedure [17] was carried out on D-glucose followed by treatment with a racemic mixture of (±)-2-octanol. The residues of the three reactions were analysed by GC-MS using the same conditions (column and carrier-gas) reported above for the analysis of alditol acetates obtained from **1**. The analysis was performed at 220°, using *myo*-inositol as int. standard.

*Acetylation of* **1**. Ascaulitoxin (**1**, 7.3 mg) dried under high vacuum for 4 h was acetylated with pyridine (300 μl) and $Ac_2O$ (300 μl) at 90° for 1 h and then at room temp. After 4 days, the reaction was stopped by addition of MeOH and the pyridine eliminated by azeotropic addition of benzene. The oily residue left by the reaction work-up, showed by TLC (silica gel, eluent C), total conversion of **1** and the presence of three main less polar derivatives ($R_f$s 0.70, 0.51 and 0.38, respectively). The mixt. was purified by a silica gel CC (eluent C) to give a hexacetyl (**4**) and two pentacetyl (**3** and **2**) derivatives of ascaulitoxin as homogeneous oils (1, 1.1 and 3 mg, respectively). These were characterised essentially by $^1H$ NMR (Table 2) and by both EI and FAB MS. **2**. HR-EI MS *m/z* (rel. int.): 571.208969 ($C_{24}H_{33}N_3O_{13}$, calculated 571.201339) $[M-2\times H_2O]^+$ (30); EI MS *m/z* (rel. int.): 529 $[M-H_2O-HOAc]^+$ (26), 527 $[M-2\times H_2O-CO_2]^+$ (74), 511 $[M-2\times H_2O-HOAc]^+$ (3), 485 $[M-2\times H_2O-CO_2-CH_2CO]^+$ (10), 467 $[M-2\times H_2O-CO_2-HOAc]^+$ (7), 452 $[M-2\times H_2O-HOAc-CH_3COO]^+$ (46), 425 $[M-2\times H_2O-CO_2-CH_2CO-HOAc]^+$ (13), 407 $[M-2\times H_2O-CO_2-2\times HOAc]^+$ (9), 331 [tetracetylglucosyl]$^+$ (60), 271 [tetracetylglucosyl–HOAc]$^+$ (50), 169 [tetracetylglucosyl–2 × HOAc–$CH_2CO$]$^+$ (95), 109 [tetracetylglucosyl–3 × HOAc–$CH_2CO$]$^+$ (100); FAB-MS *m/z* (rel. int.): 572 $[MH-2\times H_2O]^+$ (100), 554 $[M-3\times H_2O]^+$ (89), 530 $[MH-2\times H_2O-CH_2CO]^+$ (68). **3**. HR-EI MS *m/z* (rel. int.): 571.204681 ($C_{24}H_{33}N_3O_{13}$, calculated 571.2013390 $[M-2\times H_2O]^+$ (51); EI MS *m/z* (rel. int.): 527 $[M-2\times H_2O-CO_2]^+$ (83), 511 $[M-2\times H_2O-HOAc]^+$ (2), 485 $[M-2\times H_2O-CO_2-CH_2CO]^+$ (11), 467 $[M-2\times H_2O-CO_2-HOAc]^+$ (7), 452 $[M-2\times H_2O-HOAc-CH_3COO]^+$ (29), 425 $[M-2\times H_2O-CO_2-CH_2CO-HOAc]^+$ (13), 407 $[M-2\times H_2O-CO_2-2\times HOAc]^+$ (9), 331 [tetracetylglucosyl]$^+$ (41), 271 [tetracetylglucosyl–HOAc]$^+$ (78), 211 [tetracetylglucosyl–2 × HOAc]$^+$ (47), 169 [tetracetylglucosyl − 2 × HOAc–$CH_2CO$]$^+$ (100), 109 [tetracetylglucosyl–3 × HOAc–$CH_2CO$]$^+$ (53); FAB-MS *m/z* (rel. int.): 572 $[MH-2\times H_2O]^+$ (63), 554 $[MH-3\times H_2O]^+$ (100). **4**. HR-EI MS *m/z* (rel. int.): 529.185326 ($C_{22}H_{31}N_3O_{12}$, calculated 529.190774) $[M-2\times HOAc]^+$ (4.5); EI MS *m/z* (rel. int.): 649 $[M]^+$ (6), 631 $[M-H_2O]^+$ (0.7), 613 $[M-2\times H_2O]^+$ (1.4), 604 $[M-HCOO]^+$ (7), 589 $[M-HOAc]^+$ (5), 575 $[M-HCOOH-CO]^+$ (1.5), 529 $[M-2\times HOAc]^+$ (4.5), 515 $[M-HCOOH-CO-HOAc]^+$ (0.7), 469 $[M-3\times HOAc]^+$ (2.8), 455 $[M-HCOOH-CO-2\times HOAc]^+$ (13), 331 [tetracetylglucosyl]$^+$ (33), 271 [tetracetylglucosyl–HOAc]$^+$ (6), 211 [tetracetylglucosyl − 2 × HOAc]$^+$ (8), 169 [tetracetylglucosyl–2 × HOAc–$CH_2CO$]$^+$ (100), 109 [tetracetylglucosyl–3 × HOAc–$CH_2CO$]$^+$ (52); FAB-MS *m/z* (rel. int.): 650 $[MH]^+$ (100), 632 $[MH-H_2O]^+$ (18), 606 $[MH-CO_2]^+$ (30), 590 $[MH-HOAc]^+$ (16), 530 $[MH-2\times HOAc]^+$ (9), 331 [tetracetylglucosyl]$^+$ (30).

*Bioassay methods*

*Leaf-puncture.* Phytotoxic activity of culture filtrates, chromatographic frs and pure phytotoxin was tested using a leaf-puncture assay on host plants. Moreover, the phytotoxicity of pure compound was evaluated using the same assay, on several non-host plants. Test soln (15 $\mu$l) containing the desired amount of metabolites or frs, was applied to previously needle-punctured sites of detached leaves. Then, leaves were kept in moist chamber at 25° under continuous light and observed daily for symptom development.

*Cutting.* Young tomato and fat hen seedlings produced in a growth chamber, at cotyledon stage, were cut with a blade and immersed in toxin soln ($8 \times 10^{-4}$ M) under continuous light for 2 days. Cuttings were then transferred to dist. $H_2O$. Symptom development was observed after 2 days.

*Seedling.* Germinated sterile seedlings of fat hen and tomato were placed in small Petri dishes (10 seedlings for each plate) containing 2 ml of ascaulitoxin soln ($10^{-4}$ M) and kept at 25°. After 3 days, the length of rootlets was measured and toxicity expressed as percentage inhibition of root elongation compared with controls.

*Antifungal and antibiotic.* Activity on fungi and bacteria was tested on *Geotrichum candidum* and on *Pseudomonas syringae* and *Escherichia coli*, respectively, according to Ref. [23]. Ascaulitoxin was assayed up to 100 $\mu$g per disc.

*Acknowledgements*—This investigation was supported, in part, by grants from the National Research Council (CNR) and, in part, by grants from the Italian Ministry of University and Scientific and Technological Research. The authors thank Prof. A. Parente (Istituto di Biologia, Seconda Università di Napoli, Caserta, Italy) for the automatised amino-acid analyses, Dr A. Molinaro (Dipartimento di Chimica Organica e Biologica, Università di Napoli Federico II, Napoli, Italy) for the carbohydrate analysis and Dr E. Trivellone (Area della Ricerca di Napoli, CNR) for access to NMR spectrometers. We are also grateful to Mr C. Di Pinto for technical assistance. Contribution N 155 (DISCA).

## REFERENCES

1. Charudattan, R., in *Fungi in Biological Control Systems*, ed. M. N. Burge, Manchester University Press, Manchester, 1988, p. 86.
2. Kempenaar, C., PhD Thesis, Wageningen, 1995.
3. Holm, L. G., Pluckett, D. L., Pancho, J. V. and Herberger, J. P., in *The World's Worst Weed (Distribution and Biology)*, University Press of Haway, Honoloulou, 1977, p. 84.
4. Strange, R. N., in *Toxins in Plant Disease Development and Evolving Biotechnology*, ed. R. K. Upadhyay and K. G. Mukerji, Oxford & IBH Publishing Co. Pvt. Ltd., New Delhi, 1997, p. 167.
5. Strobel, G. A., Kenfield, D., Bunkers, G., Sugawara, F. and Clardy, J., *Experientia*, 1991, **47**, 819.
6. Agrawal, P. K., *Phytochemistry*, 1992, **31**, 3307.
7. Laschat, S. and Kunz, H., *Journal of Organic Chemistry*, 1991, **56**, 5883.
8. Bax, A. and Freeman, R., *Journal of Magnetic Resonance*, 1981, **44**, 542.
9. Braunshweiler, L. and Ernst, R. R., *Journal of Magnetic Resonance*, 1983, **53**, 521.
10. Pretsch, E., Clerc, T., Seibl, J. and Simon, W., in *Tables of Spectral Data for Structure Determination of Organic Compounds*, ed. W. Fresenius, J. F. K. Huber, E. Pungor, G. A. Rechnitz, W. Simon and Th. S. West, Springer-Verlag, Berlin, 1983, p. H-75, H-80, H351–H355, M-5, M-20, M-30.
11. Breitmaier, E. and Voelter, W., in *Carbon-13 NMR Spectroscopy*, VCH, Weinheim, 1987, pp. 80–84, 183–238, 380–394, 415–420.
12. Bax, A., Ikura, M., Kay, L. E., Torchia, D. A. and Tschudin, R., *Journal of Magnetic Resonance*, 1990, **86**, 304.
13. Cuberta, A., Jimeno, M. L. and Ochao, C., *Nucleoside Nucleotide*, 1987, **6**, 831.
14. Silverstein, R. M., Bassler, C. G. and Morrill, T. C., in *Spectrometric Identification of Organic Compounds*, ed. J. Wiley, New York, 1974, pp. 177–179.
15. Bax, A. and Summers, M. F., *Journal of American Chemical Society*, 1986, **108**, 2093.
16. Macura, S. and Ernst, R. R., *Molecular Physic*, 1980, **41**, 95.
17. Leontin, K., Lindberg, B. and Lonngren, J., *Carbohydrate Research*, 1978, **62**, 359.
18. Jones, J. H., in *Amino-acids, Peptides, and Protein*, Vol. 1, ed. G. T. Young, The Chemical Society Burlington House, London, 1969, p. 1; Bycroft, B. W. *ibidem*, Vol. 2, 1970, pp. 1–5 and Vol. 3, 1971, pp. 1–5.
19. Bycroft, B. W. and Higton, A. A., in *The Chemistry of Natural Products*, ed. R. H. Thomson, Blackie, Glasgow, 1985, p. 238.
20. Pinkerton, F. and Strobel, G. A., *Proceedings of the National Academy of Sciences of the United States of America*, 1987, **73**, 4007.
21. Sandford, P. A. and Conrad, H. E., *Biochemistry*, 1966, **5**, 1508.
22. Sweet, D. P., Shapiro, R. H. and Albersheim, P., *Carbohydrate Research*, 1975, **40**, 217.
23. Bottalico, A., Capasso, R., Evidente, A., Randazzo, G. and Vurro, M., *Phytochemistry*, 1990, **29**, 93.