STRUCTURE AND SYNTHESIS OF CUCUMOPINE, A NEW CROWN GALL AND HAIRY-ROOT OPINE

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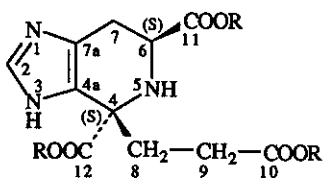
Abstract - The opine known as cucumopine 1 produced by crown gall tumours and hairy-root exhibited chemical and spectroscopic data corresponding to 4-(2-carboxyethyl)-4,5,6,7-tetrahydroimidazo[4,5-c]pyridine-4,6-dicarboxylic acid. The identification was verified by a biogenetic type synthesis from L-histidine and α-ketoglutaric acid.

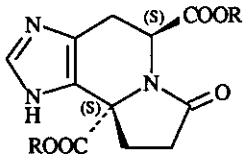
The pathogenic soil bacteria Agrobacterium tumefaciens and Agrobacterium rhizogenes induce, respectively, crown gall tumours and hairy-root in dicotyledonous plants, and divert plant metabolism towards the synthesis of specific compounds called opines[1]. Pathogenic determinants in Agrobacterium are located on closed circular DNA molecules, the Ti (A. tumefaciens) and the Ri (A. rhizogenes) plasmids. When a plant cell is infected, a segment of plasmid called T-DNA (transferred DNA), is integrated in the plant cell genome. In plant cells T-DNA codes for the biosynthesis of opines which the pathogen can utilize as specific growth substrates.

Cucumopine 1 and an acid-rearrangement product 2 were isolated[2] from grapevine crown gall tumours caused spontaneously by Agrobacterium tumefaciens, and from carrot hairy-root cultures induced by Agrobacterium rhizogenes. Purified cucumopine 1 (figure I) was obtained as an amorphous powder, $[\alpha]_D^{22}$ -33° (c = 0.37, H_2O). Its molecular formula $C_{11}H_{13}N_3O_6$ (microanalysis) was confirmed by fab mass spectroscopy MH^+ 284. The uv spectrum (H_2O) showed a band at 218 nm, indicating an unsaturated chromophore without extended conjugation. The ir spectrum exhibited

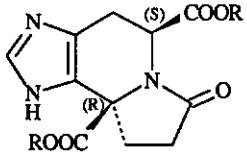
bands at 3500-3300 cm^{-1} (broad) and 1620 cm^{-1} (sharp), characteristic of carboxylic acid group absorptions. This structural feature is attested to by the presence of three carboxylic resonances in the ^{13}C nmr spectrum, at 177.7, 172.3 and 170.3 ppm (table I). The first results suggested that cucumopine 1 had a bicyclic structure with two double bonds and three carboxylic acid functions. Chemical evidence for the latter functionalities was provided by the formation of an amorphous trimethyl ester derivative 4 [diazomethane/ ether], $[\alpha]_D^{22}$ -50° (c = 0.71, $CHCl_3$), ir : 1710 cm^{-1}. The 400 MHz ^1H nmr spectrum of 4 in $CDCl_3$ showed the singlet signal of a deshielded proton at 7.65 ppm, suggesting the presence of an imidazole ring. The absence of other olefinic resonances is in agreement with an imidazole group substituted at C-4 and C-5. This proposal is corroborated by the presence in the ^{13}C nmr spectrum of an olefinic methine carbon resonance at 135.1 ppm and two quaternary olefinic carbon resonances at 129.1 and 127.7 ppm. We reasoned that a natural product containing an imidazole ring system and a third nitrogen atom, without having further unsaturation, might be derived from histidine. Indeed careful analysis of the ^1H nmr spectrum of the triester revealed signals of an ABX system, three doublets of doublets at 3.91, 2.96 and 2.76 ppm in the aliphatic part of the spectrum. The downfield double doublet at 3.91 ppm was assigned to C-6H of histidine, its downfield position being a consequence of its α-disposition to the carboxylic group and the presence of the adjacent nitrogen atom. The two other double doublets showing geminal coupling (J = 16.0 Hz) and a vicinal coupling with C-6H (J = 11.0 and 4.5 Hz), were assigned to C-7H. The values of the coupling constants proved that the carboxylic acid at C-6 was in an equatorial position. The ^{13}C nmr spectrum revealed the presence of one quaternary carbon, appearing at 61.9 ppm and two methylene carbons, at 28.6 and 32.2 ppm. These three resonances were attributed to C-4, C-8 and C-9 respectively.



1 R = H
4 R = CH_3



2 R = H
5 R = CH_3



3 R = H
6 R = CH_3

Figure I

The second substance 2 (figure I), isolated as a white amorphous powder, $[\alpha]_D^{22}$ -49° (c = 1.0, H_2O), from grapevine crown gall tumours, turned out to be an artefact which had been formed from cucumopine 1 during the acid extraction. The close relationship between the two compounds was established by the formation of 2 from 1 when the latter was heated in aqueous acid solution for 30 min.[2]. The fab mass spectrum of 2 had a molecular ion peak MH^+ 266 which corresponded to $[MH^+ - 18]$ for the compound 1. The infrared spectrum of 2 showed a strong carbonyl absorption at 1670 cm^{-1} attributed to a γ-lactam function. The ^{13}C nmr spectrum showed the presence of three carbonyl resonances. Two of them, at 174.1 and 171.8 ppm, are attributable to carboxylic acids, since the treatment of 2 with diazomethane results in the formation of a diester. The third carbonyl, at 178.7 ppm, was attributed to a lactam carbon (table I).

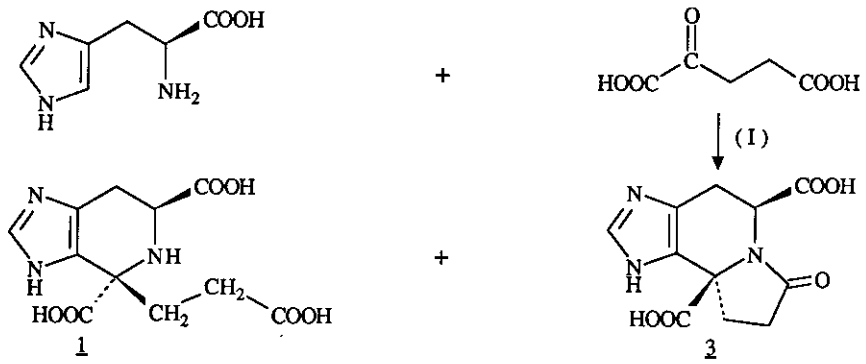
Table I : ^{13}C Nmr chemical shift values (δ ppm) for compounds 1, 2 and 3 (in D_2O) and for methyl esters 4, 5 and 6 (in $CDCl_3$).

Carbon	1	2	3	4	5	6
C-2	136.3	132.9	133.4	135.1	135.7	135.7
C-4a	126.2[a]	127.7	127.0	129.1[a]	132.9	130.8
C-4	66.1	67.8	65.3	61.9	67.2	65.7
C-6	57.0	52.7	49.4	53.3	52.8	48.3
C-7a	125.7[a]	125.4	123.8	127.7[a]	125.0	122.7
C-7	22.9	21.6	22.4	25.7	23.2	23.3
C-8	31.8[b]	28.8[a]	31.9[a]	32.2[b]	29.4[a]	31.9[a]
C-9	30.3[b]	28.1[a]	30.7[a]	28.6[b]	28.6[a]	30.8[a]
C-10	177.7[c]	178.7	177.9	174.4[c]	176.4	174.9
C-11	172.3[c]	174.1[b]	174.3[b]	173.7[c]	171.7[b]	171.9[b]
C-12	170.3[c]	171.8[b]	172.7[b]	172.0[c]	170.2[b]	170.4[b]
C-O-CH_3 ‖ O	-	-	-	52.4	53.2	52.6
	-	-	-	52.1	52.9	52.5
	-	-	-	51.3	-	-

a, b and c : the assignments may be interchanged in any vertical column.

Finally, the structures of 1 and 2 have been proved by synthesis. We based our strategy on the Pictet-Spengler reaction, which has been performed with formaldehyde[3,4], aldehydes[5,6] and

ketones[7,8]. We extended its application to the condensation of L-histidine with α-ketoglutaric acid in aqueous solution at pH 12.5 (LiOH), which afforded 1 (40 %) as white crystals, mp 205-210°C decomp. (50% aq. EtOH) and 3 (36 %) (Scheme I). Synthetic and natural cucumopines exhibited identical spectral properties (optical rotation, ms, ir, uv, nmr).



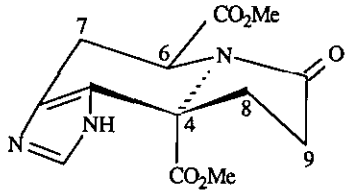
(I) Reagent : LiOH (pH 12,5), 50°C, 24 h.

Scheme I

Compound 3 was isolated as a white crystalline solid, mp 285-290°C decomp. (50% aq. EtOH), $[\alpha]_D^{22}$ +27° (c = 1.16, H_2O). The molecular formula $C_{11}H_{11}N_3O_5$ (microanalysis) was confirmed by mass spectrometry (MH^+ 266). The infrared spectrum showed absorption bands at 3300-3200 (-N-H, -O-H acid), 1710 (C = O acid) and 1670 (C = O lactam) cm^{-1}. The ^{13}C nmr spectrum (table I) of the methyl ester derivative 6 showed the presence of two methoxy groups and one carbonyl group. The coupling constant (J = 8.0 Hz) shown in ^1H nmr spectrum indicated an equatorial position for C-6H. Assuming that no inversion has occurred at C-6, compound 3 is thus the C-4 epimer of compound 2 and arises on account of the formation of a new asymmetric centre during the cyclisation reaction.

In order to confirm the relative stereochemistry of 5 and 6, NOE experiments were carried out. The absence of enhancement of the C-6H, C-8H and C-9H resonances of 5 suggested that the two carboxylic groups in C-6 and C-4 have a trans orientation, as indicated on figure II.

A detailed examination of the ^1H nmr spectrum of 6 revealed a downfield shift of the C-6H (4.6 ppm for 5 and 5.38 ppm for 6), due to the anisotropic effect of the lactam carbonyl, in agreement with a cis orientation for the two carboxylic groups. The isomeric lactams 2 and 3 exhibit somewhat different electrophoretic mobilities at pH 1.7 (Mo.g.= -0.34 for 2 and Mo.g. = -0.51 for 3 ; M.E. Tate, unpublished result). This observation is consistent with the proposed configurations since at this pH the difference in mobility is determined mainly on the extent of ionization of the 4-carboxylate group, which in turn is influenced by its proximity to the positively charged imidazole moiety.



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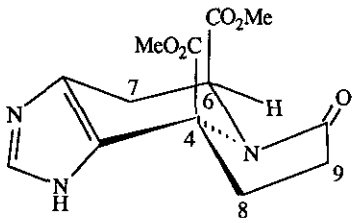

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Figure II

From these results, we propose the absolute stereochemistry depicted in figure I for 2 and 5, and therefore for cucumopine 1.

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EXPERIMENTAL

Infrared spectra (ir) were recorded on an Infracord Perkin-Elmer spectrophotometer. Ultraviolet spectra (uv) were run either in H_2O or EtOH solution on a Bausch and Lomb spectronic 505 spectrophotometer. ^1H Nuclear magnetic resonance (nmr) spectra were recorded in D_2O (HDO as an internal standard, δ = 4.65 ppm) for acid compounds, in $CDCl_3$ for ester compounds (TMS as an internal standard, δ = 0 ppm), on a Brüker WP-400 (400 MHz) instrument. ^{13}C Nmr spectra were recorded in D_2O for acid compounds, in $CDCl_3$ for ester compounds, on a Brüker WP-200 (50.33 MHz) instrument. Fab-mass spectrometry (ms) was performed on a Kratos 80RF spectrometer ; the matrix was glycerol.

Isolation of the natural products : Extraction from dry grapevine crown gall tumours (2) gave 119 mg of cucumopine 1, and 963 mg of compound 2. The compounds exhibited the following characteristics :

Cucumopine 1 : amorphous ; uv (H_2O) λ_{max}nm (log ε) 218(3.77) ; $[\alpha]_D^{22}$ -33° (c = 0.37, H_2O) ; ir (nujol) 3500-3300, 1620, 1560, 1460, 1380 cm^{-1} ; fab-ms : MH^+ 284 ; ^1H nmr (D_2O) δ ppm : 2.2-2.6 (4H, m, H-8 and H-9), 2.87 (1H, dd, J = 17.0 and 12.0 Hz, H-7), 3.21 (1H, dd, J = 17.0 and 5.0 Hz, H-7), 4.04 (1H, dd, J = 12.0 and 5.0 Hz, H-6), 8.58 (1H, s, H-2) ; ^{13}C nmr, see table I.

Compound 2 : amorphous, uv (H_2O) λ_{max}nm (log ε) 216(3.72) ; $[\alpha]_D^{22}$ -49° (c = 1.0, H_2O) ; ir (nujol) 3600-3000, 1670, 1580, 1455, 1375 cm^{-1} ; fab-ms : MH^+ 266 ; ^1H nmr (D_2O) δ ppm 2.1-2.44 (4H, m, H-8 and H-9), 2.73 (1H, dd, J = 16.5 and 5.0 Hz, H-7), 2.9 (1H, dd, J = 16.5 and 11.0 Hz, H-7), 4.26 (1H, dd, J = 11.0 and 5.0 Hz, H-6), 8.41 (1H, s, H-2) ; ^{13}C nmr, see table I

Preparation of methyl ester 4 : Natural cucumopine 1 in mixture with 2 (40 mg) in CH_3OH solution (3 ml) was esterified with a solution of diazomethane/ether (10 ml) for one hour. The reaction mixture was then concentrated in vacuo to give a crude product (46 mg) which was purified on silica gel TLC to yield triester 4 (16 mg) : amorphous ; ms m/z : M^+ 325 ; uv (EtOH) λ_{max}nm (log ε) 207(3.85), 215(3.80) ; $[\alpha]_D^{22}$ -50° (c = 0.71, $CHCl_3$) ; ir (neat) 1710, 1620, 1560, 1460, 1380 cm^{-1} ; ^1H nmr ($CDCl_3$) δ ppm 2.0-2.56 (4H, m, H-8 and H-9), 2.76 (1H, dd, J = 16.0 and 11.0 Hz, H-7), 2.96 (1H, dd, J = 16.0 and 4.5 Hz, H-7), 3.63, 3.76 and 3.83 (3-OMe, 3s), 3.91 (1H, dd, J = 11.0 and 4.5 Hz, H-6), 7.65 (1H, s, H-2) ; ^{13}C nmr, see table I.

Preparation of methyl ester 5 : Compound 2 (88mg) in CH_3OH solution (7 ml) was esterified with a solution of diazomethane/ether (20 ml) for one hour. The reaction mixture was then concentrated in vacuo to give a crude product (100 mg), which was purified on silica gel TLC to yield diester 5 (67 mg) : amorphous ; ms m/z : M^+ 293 ; uv (EtOH) λ_{max}nm (log ε) 210(3.85) ; $[\alpha]_D^{22}$ -112° (c = 1.06, $CHCl_3$), ir (neat) 1740, 1670, 1590, 1440, 1265, 1230 cm^{-1} ; ^1H nmr ($CDCl_3$) δ ppm 2.16-2.68 (4H, m, H-8 and H-9), 2.88 (1H, dd, J = 16.0 and 5.0 Hz, H-7) ; 3.26 (1H, dd, J = 16.0 and 11.5 Hz, H-7), 3.81 and 3.85 (2-OMe, 2s), 4.60 (1H, dd, J = 11.5 and 5.0 Hz, H-6), 7.65 (1H, s, H-2) ; ^{13}C nmr, see table I.

Synthesis of cucumopine 1, and compound 3 : L-histidine (1.552 g, 0.01M) was added to α-ketoglutaric acid (2.992 g, 0.02M) in water (10 ml), then the mixture was treated with LiOH (2.22 g, 0.05M). The solution (pH 12.5) was heated at 50°C during 24 h. After this, the solution was acidified to pH 2.5 with concentrated HCl. Ethanol (25 ml) was added and the solution was left at 4°C during three days to facilitate crystallisation of the product 1 as white crystals. Recrystallisation from water-EtOH 50 % yielded 3.38 g (40 %) of 1. The aqueous solution was then percolated over a Dowex 50-X8 (H^+ form) column (250 ml), cationic species

were desorbed with 1N NH_4OH, and the eluent concentrated. The solution was adjusted to pH 3 with concentrated HCl and diluted with one volume of EtOH. Product 3 precipitated as white crystals, 2.84 g (36 %).

Cucumopine 1 was identical with the natural product ($[\alpha]_D^{22}$, uv, ir, ms, nmr) mp 205-210°C decomp. Elemental analysis of $C_{11}H_{13}N_3O_6 . 1H_2O$: found C : 43.69 %, H : 4.80 %, N : 13.50 % ; calc C : 43.85 %, H : 4.31 %, N : 13.95 %.

Compound 3 : $[\alpha]_D^{22}$ +27° (c = 1.16, H_2O) ; mp 285-290°C decomp. uv (H_2O) λ_{max}nm (log ε) 218(3.98) ; ir (nujol) 3300-3200, 1710, 1670, 1580, 1460, 1380, 1350, 1220 cm^{-1} ; fab-ms : MH^+ 266 ; ^1H nmr (D_2O) δ ppm 2.03 (1H, dt, J = 13.0 and 10.0 Hz, H-9), 2.35 (1H, dd, J = 16.0 and 10.0 Hz, H-8), 2.48 (1H, dd, J = 13.0 and 10.0 Hz, H-9), 2.73 (1H, dt, J = 16.0 and 10.0 Hz, H-8), 2.83 (1H, dd, J = 16.0 and 5.0 Hz, H-7), 3.03 (1H, d, J = 16.0 Hz, H-7), 5.06 (1H, d, J = 5.0 Hz, H-6), 8.42 (1H, s, H-2) ; ^{13}C nmr, see table I ; elemental analysis of $C_{11}H_{11}N_3O_5$: found C : 49.03 %, H : 4.23 %, N : 15.18 %, calc C : 49.81 %, H : 4.18 %, N : 15.85 %.

Preparation of methyl ester 6 : Compound 3 (120 mg) in CH_3OH solution (10ml) was esterified with a solution of diazomethane/ether (30 ml) for one hour. The reaction mixture was then concentrated in vacuo to give a crude product (144 mg), which was purified on silica gel TLC to yield diester 6 (61 mg) : amorphous ; uv (EtOH) λ_{max}nm (log ε) 216(3.89), 206(3.91) ; $[\alpha]_D^{22}$ +63° (c = 1.07, $CHCl_3$) ; ms m/z M^+ 293 ; ^1H nmr ($CDCl_3$) δ ppm 2.10 (1H, dt, J = 12.0 and 9.0 Hz, H-9), 2.46 (1H, dd, J = 17.0 and 9.0 Hz, H-8), 2.77 (1H, dd, J = 12.0 and 9.0 Hz, H-9), 2.93 (1H, dt, J = 17.0 and 9.0 Hz, H-8), 2.97 (1H, dd, J = 16.0 and 8.0 Hz, H-7), 3.27 (1H, d, J = 16.0 Hz, H-7), 3.62 and 3.73 (2-OMe, 2s), 5.38 (1H, d, J = 8.0 Hz, H-6), 7.53 (1H, s, H-2) ; ^{13}C nmr, see table I.

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