

An Unusual Pentacyclic Dinitrogenous Alkaloid from *Galanthus gracilis*

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Received 04.01.2005

A minor alkaloid, namely gracilamine, was isolated from *Galanthus gracilis*. This was the first example of a pentacyclic dinitrogenous alkaloid isolated from a member of Amaryllidaceae. The structure of this alkaloid was elucidated by means of comprehensive spectroscopic methods (1D and 2D NMR, MS, UV, IR).

Key Words: Gracilamine, *Galanthus gracilis*, Pentacyclic and Dinitrogenous Alkaloids, Amaryllidaceae.

Introduction

Amaryllidaceae alkaloids possess a diverse range of interesting biological activities including antitumor, antiviral and acetylcholinesterase inhibitory activity¹⁻³. The alkaloid galanthamine provides an effective symptomatic treatment for patients with Alzheimer's disease⁴. During the course of our phytochemical research on Turkish *Galanthus* species (Amaryllidaceae), a previous investigation on the chemical constituents of *G. gracilis* resulted in the isolation of a number of new alkaloids and the establishment of a novel subgroup for the Amaryllidaceae alkaloids, called gracilines⁵⁻⁷. In the present study, we describe the isolation and characterization of gracilamine, a novel pentacyclic dinitrogenous alkaloid.

Experimental

General experimental procedures

Optical rotation: Perkin-Elmer 241 Polarimeter; UV: Perkin-Elmer 555 Spectrophotometer; IR: Perkin-Elmer 297 Infrared Spectrophotometer; 1D and 2D NMR [gs-HSQC, gs-HMBC, TOCSY (mixing time, 100 ms), NOESY, DQF-COSY]: Bruker AMX 600 Spectrometer; ESI-MS: Finnigan MAT TSQ 700; CI-MS: Finnigan MAT 90; CD: Jasco J-715 Spectropolarimeter.

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Plant material

Galanthus gracilis célak. was collected from Mount Nif, near Kemalpaşa, İzmir, in March 1995 at an altitude of 900-1500 m. A voucher specimen (No: 1192) is deposited in the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Ege University.

Extraction and isolation

Dried and powdered total plant material (5.25 kg) was extracted with ethanol (100 L) for 40 h at room temperature to afford the crude extract (681 g), which was dissolved in 2% hydrochloric acid and filtered. The acidic solution was made alkaline with 10% ammonium hydroxide and then extracted with chloroform. The evaporation of the organic solvent supplied the crude basic extract (9.58 g). During the preliminary separation through a Si gel (Merck, 70-230 Mesh) column, elution with chloroform enriched with 3% methanol afforded a 643.7 mg fraction, which was subjected to preparative TLC using benzene:chloroform:methanol (7:2:1) (saturated with ammonia vapors). The band with the R_f : 0.48 was resubjected to successive preparative TLC on Si gel plates using benzene:chloroform:ethanol (93:5:2) (saturated with ammonia vapors) and *n*-hexane: chloroform (1:1) (saturated with ammonia vapors) as solvent systems to yield 3.8 mg of gracilamine (**1**); R_f 1: 0.28; 2: 0.60.

Gracilamine (1): Colorless amorphous solid. $[\alpha]_D = +21.8^\circ$ (c 0.133, MeOH). CD (MeOH) nm (lg ϵ) = 313 (0), 294 (- 1.115), 231 (+ 0.895), 224 (- 0.004), 218 (- 1.099), 212 (+ 0.058), 201 (+ 9.431). UV (MeOH) λ_{max} nm (lg ϵ) = 234 sh (4.01), 268 (3.37), 295 (3.71). IR (CHCl₃) ν = 2960, 1725 sh, 1715, 1695 sh, 1600, 1500, 1475, 1455 sh, 1410, 1370, 1350, 1330, 1320, 1265, 1215, 1145, 1125, 1095, 1040, 940, 920, 860 cm⁻¹. ¹H NMR (600 MHz, CD₃OD): δ = 6.86 (1 H, s, H-10), 6.68 (1 H, s, H-13), 5.95 (1 H, d, J = 1.1 Hz, OCH₂O), 5.93 (1 H, d, J = 1.1 Hz, OCH₂O), 4.67 (1 H, d, J = 8.0 Hz, H-9a), 3.96 (1 H, d, J = 7.2 Hz, H-17_a), 3.94 (1 H, d, J = 7.2 Hz, H-17_b), 3.50 (1H, bt, H-6), 3.19 (1H, m, H-2_a), 3.15 (1H, dd, J = 8.0, 10.0 Hz, H-4), 2.92 (1H, bt, H-7a), 2.65 (1H, m, H-2_b), 2.54 (3H, s, NMe), 2.29 (1H, dd, J = 8.1, 10.0 Hz, H-5), 2.05 (1H, m, H-3_a), 1.97 (1H, dd, J = 6.8, 14.0 Hz, H-19_a), 1.95 (1H, ddd, J = 3.6, 6.7, 13.5 Hz, H-7_{eq}), 1.89 (1H, m, H-3_b), 1.72 (1H, m, H-20), 1.64 (1H, dd, J = 5.6, 14.0 Hz, H-19_b), 1.61 (1H, m, H-7_{ax}), 1.21 (3H, t, J = 7.2 Hz, Me-18), 1.01 (3H, d, J = 6.7 Hz, Me-21), 0.86 (3H, d, J = 6.7 Hz, Me-22) ppm. ¹³C NMR (150 MHz, CD₃OD): δ = 175.8 (C-16), 150.2 (C-12), 149.2 (C-11), 144.8 (C-13a), 136.5 (C-9b), 106.0 (C-10), 103.6 (C-13), 102.8 (OCH₂O), 74.1 (C-8), 71.3 (C-7a), 67.7 (C-9a), 66.4 (C-6), 62.2 (C-17), 58.6 (C-5), 55.9 (C-4), 55.6 (C-2), 54.6 (C-3a), 48.0 (C-19), 45.1 (C-3), 40.8 (NMe), 34.6 (C-7), 26.4 (C-20), 24.9 (C-21), 23.7 (C-22), 14.2 (C-18) ppm. ESI-MS: m/z = 443 [M+H⁺]. CI-MS: m/z = 443 [M+H⁺].

Results and Discussion

The initial examination of the ¹H NMR spectrum of the dextrorotatory compound, gracilamine (**1**) (Figure 1), showed characteristic singlets associated with the para-oriented aromatic protons at δ 6.86 and 6.68, which, together with 2 doublets for the methylenedioxy group at δ 5.95 and 5.93, established the substitution pattern of the aromatic ring. The signals in the aliphatic region indicated the presence of 6 methines (δ 4.67, 3.50, 3.15, 2.92, 2.29, 1.72), and 5 methylenes (δ 3.96, 3.94; 3.19, 2.65; 2.05, 1.89; 1.97, 1.64; 1.95, 1.61), along with 4 methyls (δ 2.54, 1.21, 1.01, 0.86), the most deshielded signal of which pointed to the presence

of a *N*-methyl group.

The multiplicities of the 25 carbons accounted for in the ^{13}C NMR spectrum were determined by an HSQC experiment that allowed the correlation of the protonated carbons with the corresponding hydrogens (Table). One of the most striking signals in the ^{13}C NMR spectrum was the carbonyl resonance at δ 175.8 associated with an ester group, the presence of which was supported by a strong absorption (1715 cm^{-1}) in the carbonyl stretching frequency area of the IR spectrum. Another noteworthy signal was the carbon singlet at δ 54.6, hinting at a spiro center.

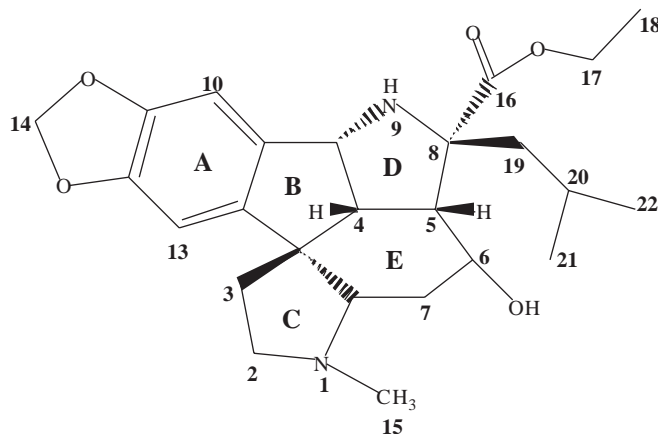


Figure 1. Gracilamine (**1**).

In agreement with the 1D NMR findings, the $[\text{M}+\text{H}^+]$ ion at m/z 443 in the ESI-MS and CI-MS experiments revealed that compound **1** had the molecular formula $\text{C}_{25}\text{H}_{34}\text{N}_2\text{O}_5$, which suggested that **1** incorporated 2 nitrogen atoms.

Since the 1D NMR and MS findings conformed to none of the so-far described subgroups of the Amaryllidaceae alkaloids⁸, extensive 2D NMR experiments were performed.

Initially, 4 partial structures (Figure 2) were deduced from the extensive analyses of 2D NMR data derived from the ^1H , ^1H DQF-COSY, TOCSY, HSQC and HMBC spectra (Table). Fragment **a** has already been established by 1D NMR data, as described above.

In the ^1H NMR spectrum, the relatively deshielded doublet at δ 4.67 ($J= 8.0$ Hz) coupled with the signal at δ 3.15 ($J= 8.0, 10.0$ Hz), which in turn correlated with the signal at δ 2.29 ($J= 10.0$ Hz). A coupling of $J= 8.1$ Hz was also observed between this proton (H-5) and a methine proton resonating at δ 3.50 (H-6). The relatively downfield chemical shift of the latter suggested the presence of a secondary alcohol. The 3 spin system, consisting of the methylene group at δ 1.95 and 1.61 (H-7_{eq} and H-7_{ax}) and the methine proton at δ 2.92 (H-7a), was connected to the above-mentioned spin system by the relevant correlations in the ^1H , ^1H DQF-COSY. Moreover, the TOCSY correlations confirmed that all of these hydrogens were involved in the same spin system, which was denoted as fragment **b**.

The correlations between the 2 methyl protons resonating at δ 1.01 (Me-21) and 0.86 (Me-22), the methine proton resonating at δ 1.72 (H-20) and the methylene group at δ 1.97 (H-19_a) and 1.64 (H-19_b) in the ^1H , ^1H DQF-COSY and TOCSY spectra were indicative of an isobutyl moiety.

The couplings of methylene doublets resonating at δ 3.96 (H-17_a) and 3.94 (H-17_b) ($J= 7.2$ Hz) and the methyl group resonating at δ 1.21 (Me-18) ($J= 7.2$ Hz), as well as the relatively downfield chemical

shifts of the aforementioned methylene hydrogens, suggested the presence of an ethoxy group, which was confirmed by the prominent correlations observed in the ^1H , ^1H DQF-COSY and TOCSY experiments. In the HMBC experiment, $^2J_{CH}$ correlations from H-17_a (δ 3.96) and H-17_b (δ 3.94) to the methyl carbon at δ 14.2 (C-18) and $^3J_{CH}$ correlations to the quaternary carbon resonating at δ 175.8 (C-16) revealed that the ethoxy group was in the form of an ethyl ester. $^2J_{CH}$ correlations from the methylene signals of the isobutyl moiety resonating at δ 1.97 (H-19_a) and 1.64 (H-19_b) to the quaternary carbon resonating at δ 74.1 (C-8) and $^3J_{CH}$ correlations to the quaternary carbon of the ethyl ester resonating at δ 175.8 (C-16) assigned the relative positions of the isobutyl moiety and the ethyl ester, which together established fragment **c**.

Table. 1D and 2D NMR data of gracilamine (**1**), δ_H and δ_C in ppm.

H	^1H NMR	HSQC	HMBC	^1H , ^1H DQF COSY	TOCSY	NOESY
2 _a	3.19	55.6	-	H-2 _b , H-3 _a , H-3 _b	H-2 _b , H-3 _a , H-3 _b	H-2 _b , H-3 _a , H-3 _b , NMe
2 _b	2.65	55.6	-	H-2 _a , H-3 _a , H-3 _b	H-2 _a , H-3 _a , H-3 _b	H-2 _a , H-3 _a , H-3 _b , H-7 _a , H-13
3 _a	2.05	45.1	C-2, C-3 _a , C-7 _a , C-13 _a	H-2 _a , H-2 _b , H-3 _b	H-2 _a , H-2 _b , H-3 _b	H-2 _a , H-2 _b , H-3 _a , H-4
3 _b	1.89	45.1	C-2, C-3 _a , C-7 _a , C-13 _a	H-2 _a , H-2 _b , H-3 _a	H-2 _a , H-2 _b , H-3 _a	H-2 _a , H-2 _b , H-3 _b
4	3.15	55.9	C-3, C-3 _a , C-5, C-6, C-8, C-9 _a , C-9 _b , C-13 _a	H-5, H-9 _a	H-5, H-6, H-7 _{ax} , H-7 _{eq} , H-7 _a , H-9 _a	H-3 _a , H-3 _b , H-5, H-9 _a , H-19 _b
5	2.29	58.6	C-3 _a , C-6, C-8, C-9 _a , C-16, C-19	H-4, H-6	H-4, H-6, H-7 _{ax}	H-4, H-6, H-19 _a , H-19 _b
6	3.50	66.4	-	H-5, H-7 _{ax} , H-7 _{eq}	H-4, H-5, H-7 _{ax} , H-7 _{eq} , H-7 _a , H-9 _a	H-5, H-7 _{ax} , H-7 _{eq} , H-7 _a
7 _{ax}	1.61	34.6	C-6, C-7 _a	H-6, H-7 _{eq} , H-7 _a	H-4, H-5, H-6, H-7 _{eq} , H-7 _a , H-9 _a	H-6, H-7 _{eq} , H-7 _a , NMe
7 _{eq}	1.95	34.6	C-3 _a , C-5, C-6, C-7 _a	H-6, H-7 _{ax} , H-7 _a	H-4, H-5, H-6, H-7 _{ax} , H-7 _a , H-9 _a	H-6, H-7 _{ax} , H-7 _a , NMe
7 _a	2.92	71.3	NMe	H-7 _{ax} , H-7 _{eq}	H-4, H-5, H-6, H-7 _{ax} , H-7 _{eq} , H-9 _a	H-2 _b , H-3 _b , H-6, H-7 _{ax} , H-7 _{eq} , H-13, NMe
9 _a	4.67	67.7	C-3 _a , C-5, C-8, C-9 _b , C-10, C-13 _a	H-4	H-4, H-5, H-6, H-7 _{ax} , H-7 _{eq} , H-7 _a	H-4, H-10, H-19 _b , H-20, Me(21)
10	6.86	106.0	C-9 _a , C-9 _b , C-11, C-12, C-13, C-13 _a	-	H-13	H-9 _a
13	6.68	103.6	C-3 _a , C-9 _b , C-10, C-11, C-12	-	H-10	H-2 _b , H-3 _b , H-7 _a , NMe
OCH ₂ O	5.95; 5.93	102.8	C-11, C-12	-	-	-
NMe	2.54	40.8	C-2, C-7 _a	-	-	H-2 _a , H-7 _{ax} , H-7 _{eq} , H-7 _a
17 _a	3.96	62.2	C-16, C-18	Me(18)	Me(18)	Me(18), Me(22)
17 _b	3.94	62.2	C-16, C-18	Me(18)	Me(18)	Me(18), Me(22)
Me(18)	1.21	14.2	C-17	H-17 _a , H-17 _b	H-17 _a , H-17 _b	H-17 _a , H-17 _b
19 _a	1.97	48.0	C-5, C-8, C-16, C-20, C-21, C-22	H-19 _b , H-20	H-19 _b , H-20, Me(21), Me(22)	H-5, H-19 _b , H-20, Me(21), Me(22)
19 _b	1.64	48.0	C-5, C-8, C-16, C-20, C-21, C-22	H-19 _a , H-20	H-19 _a , H-20, Me(21), Me(22)	H-4, H-5, H-9 _a , H-19 _a , Me(21), Me(22)
H-20	1.72	26.4	C-8, C-19, C-21, C-22	H-19 _a , H-19 _b , Me(21), Me(22)	H-19 _a , H-19 _b , H-20, Me(22)	H-5, H-9 _a , H-19 _a , Me(21), Me(22)
Me(21)	1.01	24.9	C-19, C-20, C-22	H-20	H-19 _a , H-19 _b , H-20, Me(22)	H-9 _a , H-19 _a , H-19 _b , H-20, Me(22)
Me(22)	0.86	23.7	C-19, C-20, C-21	H-20	H-19 _a , H-19 _b , H-20, Me(21)	H-17 _a , H-17 _b , H-19 _a , H-20, Me(21)

The 2 methylene groups at δ 3.19 (H-2_a), 2.65 (H-2_b) and δ 2.05 (H-3_a) and 1.89 (H-3_b), showing prominent correlations in the ^1H , ^1H DQF-COSY and TOCSY experiments, established the presence of a 4 spin system. The $^3J_{CH}$ correlations from the *N*-methyl hydrogens to the carbon resonating at δ 55.6 (C-2) established the structural fragment **d**.

In the HMBC experiment, besides the $^3J_{CH}$ correlations from δ 4.67 (H-9_a) to the carbons resonating at δ 106.0 (C-10) and δ 144.8 (C-13_a), $^2J_{CH}$ correlations to C-9_b (δ 136.5) and $^3J_{CH}$ correlations from H-4 (δ 3.15) to C-13_a (δ 144.8) gave rise to the connectivity of partial structures **a** and **b**. In addition, HMBC correlations of the hydrogens at δ 4.67, 3.15 and 2.29 to the quaternary carbon at δ 74.1 (C-8) suggested the connectivity of partial structures **b** and **c** through a nitrogen atom, the presence of which was confirmed

by the relatively deshielded chemical shift of δ 4.67 (H-9a) and the quaternary carbon resonating at δ 74.1 (C-8).

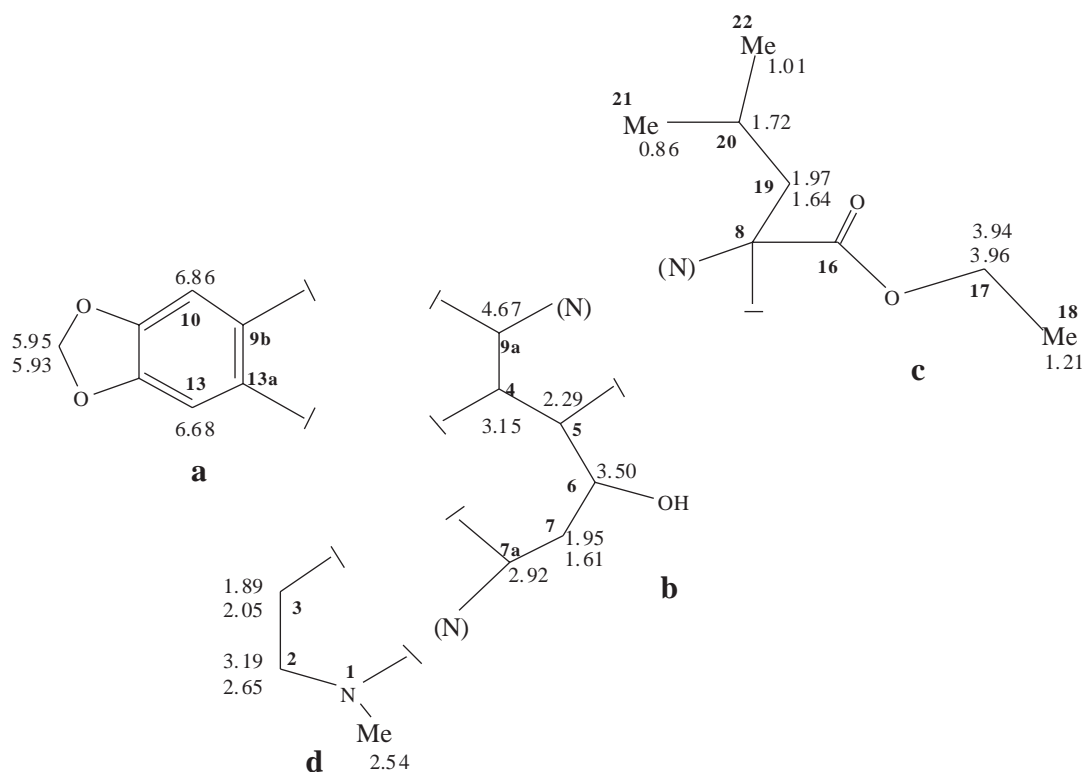


Figure 2. Structural fragments of **1** deduced from 2D-NMR measurements.

In addition to the prominent HMBC correlation between the hydrogen resonating at δ 1.95 (H-7_{eq}) and the carbon resonating at 54.6 (C-3a), $^3J_{CH}$ correlations from the methine hydrogen at δ 2.92 (H-7a) to the *N*-methyl carbon and from H-4 (δ 3.15) to C-3 (δ 45.1), 2 bond and 3 bond connectivities from the methylene hydrogens (δ 2.05 and 1.89) (H-3_a and H-3_b) to the quaternary carbons resonating at δ 54.6 (C-3a) and δ 144.8 (C-13a), respectively, allowed us to construct the pentacyclic framework of the molecule incorporating a spiro center at δ 54.6 (C-3a).

The relative stereochemistry of **1** was resolved by 2D NMR NOESY data. The strong spatial relationships of δ 4.67 (H-9a) and 3.15 (H-4), as well as of δ 3.15 (H-4) and 2.29 (H-5) indicated the co-facial relationship between 3 hydrogens. A prominent mutual spatial interaction between δ 6.86 (H-10) and 4.67 (H-9a) was also observed. The other aromatic singlet at δ 6.68 (H-13) displayed prominent spatial interactions with the hydrogens resonating at δ 2.65 (H-2_b), 1.89 (H-3_b) and 2.92 (H-7a). In turn, H-2_b (δ 2.65) displayed substantial correlations with H-7a (δ 2.92) and H-3_b (δ 1.89). On the other hand, reciprocating interactions were observed between H-2_a (δ 3.19) and H-3_a (δ 2.05), as well as between H-3_a (δ 2.05) and H-4 (δ 3.15). From these findings it was deduced that H-4 (δ 3.15), H-5 (δ 2.29) and H-9a (δ 4.67) occupied the β face of the molecule. The spatial arrangement of ring C was almost perpendicular to the vertical plane established by the rings A/B. Thus, the hydrogens of the 2 methylene groups occupying positions 2 and 3 of ring C were protruding in front of the plane established by the rings A/B. A mutual correlation between H-4 (δ 3.15) and H-7_{ax} (δ 1.61), a strong interaction between H-7_{eq} (δ 1.95) and H-7a

(δ 2.92) and key NOESY correlations from H-6 (δ 3.50) to H-5 (δ 2.29) and to H-7_{eq} (δ 1.95) were clearly observed, which indicated that ring E adopted a half-chair conformation.

The relative conformation at C-8 (δ 74.1), to which the ester and the isobutyl moiety are attached, was also deduced from the relevant NOESY correlations. In this context, worthy of mention were the spatial interactions between H-5 (δ 2.29) and H-19_a (δ 1.97), H-19_b (δ 1.64), H-20 (δ 1.72) and between H-9a (δ 4.67) and H-19_b (δ 1.64). The correlations from the methylene hydrogens of the ethyl ester to only Me(18) (δ 1.21) and Me(22) (δ 0.86) and to no other hydrogens of the parent molecule confirmed that the ester moiety was oriented toward the back of the plane established by the rings A/B.

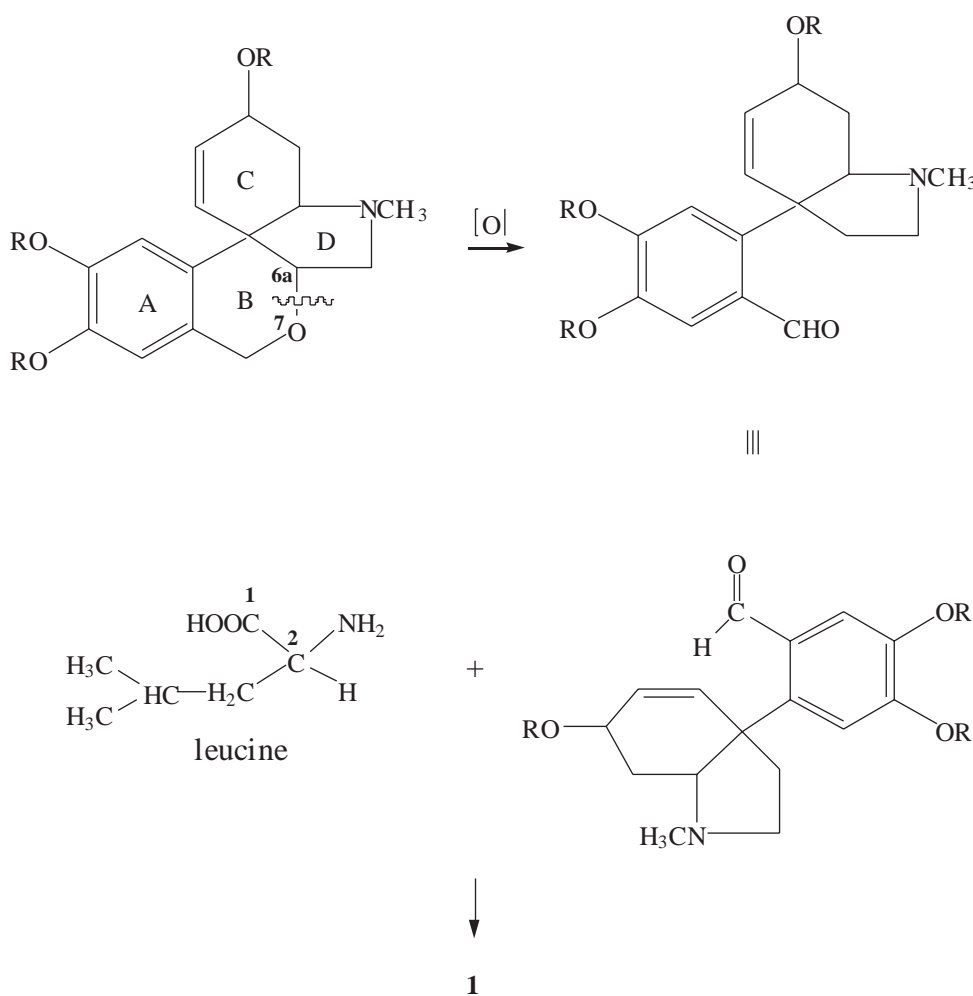


Figure 3. A probable biogenetic pathway proposed for gracilamine (**1**).

A tentative postulation for the formation of the pentacyclic skeleton involves the reaction of a crinine- or tazettine-type alkaloid with the amino acid leucine, which provides the second nitrogen atom. The aldehyde, formed by the enzymatic oxidation resulting in the cleavage of the C-6a,7 bond in ring B of the alkaloid⁹, forms one of the reactive centers. The double bond of ring C of the alkaloid and the active hydrogen at C-2 of leucine are thought to be other groups taking part in the formation of the pentacyclic structure. Further esterification of the carboxylic group affords compound **1** (Figure 3). This suggestion should be verified by future synthetic studies or labeled feeding experiments.

The occurrence of this unusual pentacyclic dinitrogenous alkaloid in *G. gracilis* once again proves that this species is a promising source for alkaloids with interesting structural diversities.

Acknowledgments

This work was supported by the Scientific and Technical Research Council of Turkey (TÜBİTAK) and the Ege Science and Technology Center (EBİLTEM) (Project No 2001/BIL/019).

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