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CLITIDINE, A NEW TOXIC PYRIDINE NUCLEOSIDE FROM *CLITOCYBE ACROMELALGA*

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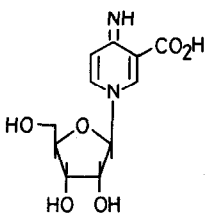
Abstract—From a poisonous mushroom, *Clitocybe acromelalga*, a new nucleoside clitidine (1) was isolated as a toxic principle. The structure was deduced to be 1 from spectral data and chemical degradation studies. Synthesis of 1 through condensation of methyl 4-aminonicotinate with 3, 5-di-*O*-benzoyl-D-ribofuranosyl chloride confirmed the structure, including absolute configuration.

A poisonous mushroom, *Clitocybe acromelalga* (Japanese name: Dokusasako), is found only in Japan. Its poisonous property has been described in more than twenty papers in the past 100 yr. Poisoning is very unique, that is, accidental ingestion of the fungus causes a violent pain and red coloration in the fingers and toes after a period of several days and the pain continues over 2 weeks. The symptoms are similar to erythromelalgia or acromelalgia.

These characteristic physiological properties prompted us to study the chemical constituents of this fungus. It is known that the water extract exhibits a significant lethal effect in mice,[1] so that fractionation was carried out by the guide of this effect and a new pyridine nucleoside, named clitidine 1, was isolated. We herein describe the isolation, structural elucidation and synthesis of clitidine.[2]

A number of pyridine nucleosides have been synthesized in order to examine their physiological activities.[3–14] However, to our knowledge clitidine is the first example of the natural pyridine nucleoside.



clitidine 1

Isolation. The water extract of fruit bodies was treated with acetone to yield precipitates which were dialized against water to eliminate high molecular weight substances. The dialyzate was applied on a column of active charcoal and eluted stepwise with ethanol and water to give a crystalline mass. Recrystallization of the product from water afforded pure crystals of clitidine. Yield amounted to 190 mg from 1 kg of fruit bodies.

Structural elucidation. The molecular formula of clitidine was determined to be $C_{11}H_{14}N_2O_6$ by elemental analysis and FD-MS [*m/e* 271 $(M+H)^+$]. In the ^1H NMR spectrum, three signals at 3.91 (2H, bs), 4.30 (3H, bs), 5.70 (1H, d, $J = 4.5$ Hz) were thought to be attributable to a pentose, in particular, the doublet at 5.70 was assignable to the anomeric proton. The ^{13}C NMR spectrum also exhibited the signals due to a pentose at δ 63.6 (t), 72.9 (d), 78.7 (d), 89.1 (d) and 99.9 (d) ppm. The presence of the pentose moiety was further supported by the chemical ionization mass spectrum, which showed fragment peaks at *m/e* 133, 115 (133–H_2O) and 97 (133–$2H_2O$) characteristic of ribonucleosides.[15] Positive *cis*-diol test[16] for clitidine suggested that the pentose had a ribofuranose structure, which was confirmed by synthesis described later.

The high-resolution mass spectrum showed an intense signal at *m/e* 138.0423 ($C_6H_6N_2O_2$) as the base peak which suggested a formula $C_6H_5N_2O_2$ to the base portion of the compound. In the ^1H NMR spectrum signals due to three aromatic protons were observed at 6.92 (d, $J = 7$ Hz), 8.11 (dd, $J = 1.5$, 7 Hz), 8.72 (d, $J = 1.5$ Hz). The ^{13}C NMR spectrum exhibited three doublets (δ 114.8, 141.2, 145.1) and three singlets (δ 117.5, 162.3, 172.4). These data suggested the presence of a 2, 4- or 3, 4-disubstituted pyridine nucleus. It turned out that one of the substituents was a carboxyl group by the ^{13}C NMR peak at 172.4, and the IR band at 1660 cm^{-1}, and the fragmentation peak by loss of CO_2H in the high-resolution mass spectrum (*m/e* 138.0423, $C_6H_6N_2O_2$ and *m/e* 93.0476, $C_5H_5N_2$). The elemental composition revealed that the other substituent was an amino group. The chemical shifts of aromatic protons and carbons indicated that the carboxyl group was placed at position-3 and the amino group was at position 4 or 6 that is, the aromatic moiety was 4 or 6-aminonicotinic acid. Of these alternatives, the former was verified by chemical degradation studies. Clitidine was heated in a sealed tube for 16 h with concd. HCl to give 4-aminonicotinic acid hydrochloride[17] in quantitative yield.‡ Therefore clitidine is most probably an *N*-ribofuranoside of 4-aminonicotinic acid such as 1.

Syntheses. In order to verify the suggested structure, synthesis of clitidine 1 and its amide 5 was attempted (Scheme 1). Methyl 4-amino-nicotinate 2[18] was smoothly condensed with 3, 5-di-*O*-benzoyl-D-ribofuranosyl chloride[19] in CH_2Cl_2 at room temp. to give blocked nucleoside 4 in 83% yield.§ Subsequent hydrolysis of 4

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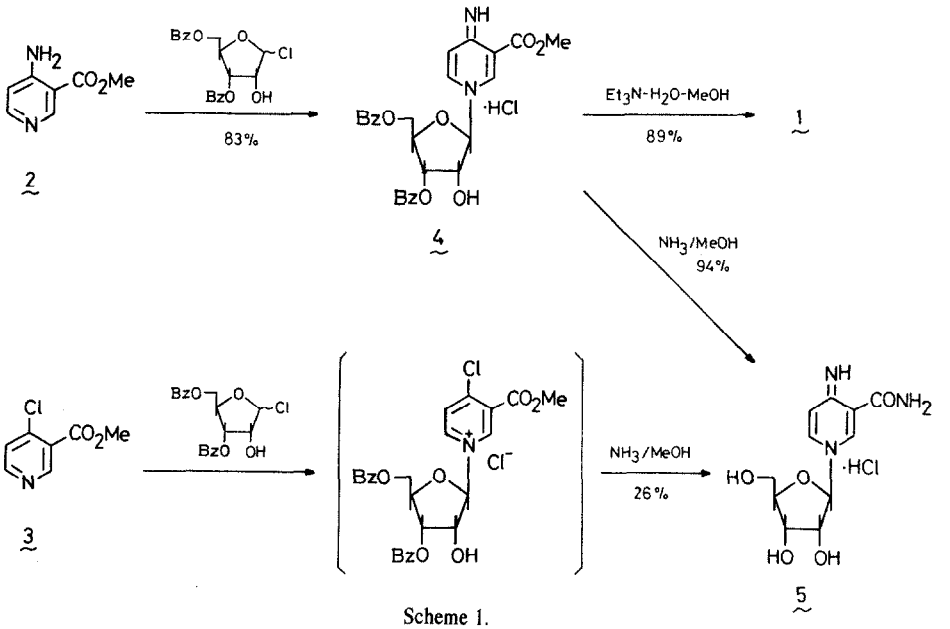
‡Clitidine was considerably resistant to acid hydrolysis. For example, heating for 16 h in 6N HCl gave no detectable changed material.

§Initially, condensation of methyl 4-aminonicotinate 2 with 2, 3, 5-tri-*O*-acetyl-D-ribofuranosyl bromide[20] was attempted, but in this case no reaction occurred. It was reported that the reaction of 3, 5-di-*O*-benzoyl-D-ribofuranosyl chloride with nicotinamide was rapid and quantitative whilst retaining the advantage of stereochemistry.[6]



Scheme 1.

with Et_3N-MeOH-H_2O yielded clitidine **1** in 89% yield. Clitidine thus obtained was identical with the natural product in chromatographic behavior and spectral data including optical rotation. As a result of this synthesis, the sugar portion of clitidine was unambiguously determined as D-ribose.

In the above condensation reaction, there are two possible ribosylation site, the ring nitrogen and the nitrogen substituted at C-4. The alkylation at the ring nitrogen was verified by the following series of reactions. Methyl 4-chloronicotinate[18] **3** was ribosylated analogously to the above condensation, and the resulting blocked nucleoside was, without purification, treated with methanolic ammonia to give a nicotinamide riboside **5** in 26% yield. On the other hand, treatment of **4** with methanolic ammonia afforded the same nucleoside **5** in 94% yield. The result clarified unambiguously the structure of clitidine as shown by formula **1**.

The synthesis implied β-configuration of the anomeric position since the condensation reaction of 3, 5-di-*O*-benzoyl ribofuranosyl chloride with a base was reported to proceed stereoselectively to give exclusively a β-anomer.[6] The β-configuration was confirmed by spectroscopic evidence using the 2, 3-*O*-isopropylidene derivative of clitidine **6**, which was obtained in 65% yield by treatment with acetone in the presence of $HClO_4$. In the ^1H NMR spectrum of the 2, 3-*O*-isopropylidene derivative (in DMSO-d_6), the chemical shift-difference ($\Delta\delta$) between two Me groups in the isopropylidene group was 0.25 ppm, and the signal of H-4′ appeared as a multiplet. According to an empirical rule found by Imbach *et al.*[21–26] this result clearly showed that the anomeric configuration of clitidine was β.[27]

Thus the structure of clitidine was determined to be 3-carboxy-4-imino-1-(β-D-ribofuranosyl)-1, 4-dihydropyridine **1**.†[28]

Biological activity. Toxicity of clitidine (LD_{50}) was determined as about 50 mg/kg by intraperitoneal injection in mice. However, no remarkable observation has been obtained from other various physiological experiments for animals so far. Further investigations are now in progress.

EXPERIMENTAL

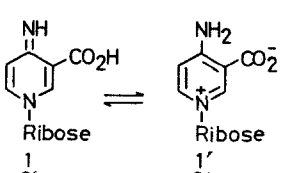
All m.ps are uncorrected. IR absorption spectra were recorded on a JASCO model IR-S spectrophotometer. ^1H NMR and ^{13}C NMR spectra were recorded on a Hitachi R-20B (60 MHz) and JEOL JNM-FX 100 (25.0 MHz) respectively. TMS in $CDCl_3$, CD_3OD, DMSO-d_6 and 3-(trimethylsilyl)-1-propane-sulfonic acid sodium salt (DSS) in D_2O were employed as internal standards.

EI, CI mass spectra (MS) and high-resolution mass spectra (Exact Mass) were measured on a JEOL D300 instrument. Field-desorption mass spectra (FD-MS) were measured on a JEOL model JMF-01SG-2 instrument. Optical rotations were taken with a JASCO model DIP-SL automatic polarimeter. Ultraviolet (UV) spectra were obtained using a Hitachi model 200-10 spectrophotometer. Circular dichroism (CD) spectra were recorded on a JASCO J-40 spectropolarimeter.

Ascending paper chromatography (PPC) was carried out on Whatman 3MM filter paper using the following solvents; (a) n-BuOH–AcOH–water (4:1:5), (b) Py–AcOEt–water (10:4:3), (c) n-BuOH–H_2O (saturated), (d) i-PrOH–water (3:1), and visualised by a UV lamp or cis-diol test.[16] Paper electrophoresis (PEP) was performed on Whatman 3MM filter paper using a model 20-TR apparatus (MS-kiki Co. Ltd.). Separations were obtained either at pH 4.6 (Py–AcOH–water, 3:3:994 by volume) or at pH 9.2 (0.05M Borax), 600V, 1.5 h unless otherwise stated. Active charcoal for chromatography was purchased from Wako Pure Chemical Ltd.

Fruit bodies of *Clitocybe acromelalga* were collected at Nagaoka city, Niigata-ken, Japan, and frozen upon collection, and stored at −20°.

†Among two possible tautomers **1** and **1′**, it was shown from UV and NMR spectral data that the tautomer **1** was predominant.[27]



Isolation of clitidine **1**

Frozen fruit bodies (5.4 kg) were extracted with water (10 l.) at 4° overnight. The extract was filtered off, and the residue was re-extracted twice. The combined extract was concentrated under reduced pressure to 600 ml (concentration was carried out below 35° throughout all the procedure). To this turbid soln was added acetone (2.4 l.) to give a brownish precipitate. After storage at 4° for 1–2 days to complete the precipitation, the supernatant was separated by decantation. The precipitate was dissolved in water (250 ml), and dialyzed against water (6 l.) at 4° overnight. Nondialyzate was concentrated and dialyzed two more times. The combined dialyzate was evaporated under reduced pressure and the residue (82 g) was applied to a column of charcoal (82 g, packed with water). After elution with water (4 l.), stepwise elution with EtOH and water was carried out (2.5, 5, 10, 30% aq EtOH, each 4 l.). Crude crystals of clitidine was obtained from 30% aq EtOH fraction, and recrystallization twice from water gave pure samples (1 g). The entire process was repeated two more times to yield a total of 3 g of clitidine. m.p. 189–191°. $[\alpha]_D^{24°}$ −50.6° (*c* 0.88, H_2O). Analysis, Found: C, 46.33; H, 5.42: N, 9.80, calc. for $C_{11}H_{14}N_2O_6 \cdot H_2O$: C, 45.83; H, 5.60; N 9.72%. FD-MS: *m/e* 271 $(M+H)^+$. CI-MS: *m/e* 139 $(base+2H)^+$, 133 (pentose), 121 (*m/e* 139-H_2O), 115 (pentose-H_2O), 97 (pentose-2H_2O), 93 (*m/e* 121-CO). Exact Mass: *m/e* 138.0423 (100%, $C_6H_6N_2O_2$), 121.0347 (13%, $C_6H_5N_2O$), 93.0476 (13%, $C_5H_5N_2$), IR (nujol): 3300–2200, 1660, 1585, 1065, 1030 cm^{-1}. UV: 271 nm (logϵ 4.09, H_2O), 267 (logϵ 4.16, pH 2), 271 (logϵ 4.18, pH 12). ^1H NMR (D_2O): 3.91 (2H, bs, 5'-H), 4.30 (3H, bs, 2', 3', 4'-H), 5.70 (1H, d, J = 4.5 Hz, 1'H), 6.92 (1H, d, J = 7 Hz, 5-H), 8.11 (1H, dd, J = 1.5, 7 Hz, 6-H), 8.72 (1H, d, J = 1.5 Hz, 2-H). CD: $[\theta]_{273}^{H_2O}$ −3100. PPC: solvent (a) Rf 0.50; (b) 0.55; (c) 0.10. PEP: pH 4.6, −1 cm; pH 9.2, +3 cm.

Acid hydrolysis of clitidine

A soln of clitidine (20 mg) in conc HCl (2 ml) was heated at 130° for 16 h in a sealed tube. The reaction mixture was filtered through celite, and the filtrate was evaporated to dryness. The crystalline residue was applied to a column of charcoal (0.5 g, packed with water). A 30% aq acetone eluate furnished crystals of 4-aminonicotinic acid hydrochloride (10 mg, 100%). The IR and ^1H NMR spectra were superimposable on those of synthetic samples.[17] IR (nujol): 3500, 3380, 1698, 1655, 1230, 1172, 1112 cm^{-1}. ^1H NMR (D_2O): 6.98 (1H, d, J = 7 Hz, 5-H), 8.03 (1H, dd, J = 1.5, 7 Hz, 6-H), 8.74 (1H, d, J = 1.5 Hz, 2-H).

3-*Methoxycarbonyl*-4-*imino*-1-(3, 5-*di*-O-*benzoyl*-β-*D*-*ribofuranosyl*)-1, 4-*dihydropyridine hydrochloride* **4**

Methyl 4-aminonicotinate (400 mg, 2.63 mmol) was dissolved in dry CH_2Cl_2 (45 ml) with warming. To this solution, after cooling to room temp and before beginning crystallization, was added 3, 5-di-*O*-benzoyl-D-ribofuranosyl chloride (1 g, 2.65 mmol) and the mixture was kept at room temp overnight. The solvent was removed and the crystalline residue was recrystallized from MeOH–ether (1:3) to give **4** (1.2 g, 83%). m.p. 160–161°. $[\alpha]_D^{24°}$ −138° (*c* 0.79, $CHCl_3$). IR (nujol): 3400, 1730, 1665, 1270, 1100 cm^{-1}. UV: 269 nm (logϵ 4.41, EtOH). EI-MS: *m/e* 152 (base), 138 (base-CH_2), 105 (benzoyl). ^1H NMR (CD_3OD): 3.72 (3H, s, $-CO_2CH_3$), 4.5–5.0 (4H, m, 2', 4', 5'-H), 5.64 (1H, dd, J = 2, 5 Hz, 3'-H), 5.99 (1H, d, J = 6 Hz, 1'-H), 7.10 (1H, d, J = 7 Hz, 5-H), 7.2–8.2 (10H, m, benzoyl), 8.28 (1H, dd, J = 1.5, 7 Hz, 6-*H*), 8.92 (1H, d, J = 1.5, 2-H). Analysis, Found: C, 58.40; H, 4.66; N, 5.12; Cl, 6.50; Calc. for $C_{26}H_{25}O_8N_2Cl$: C, 59.04; H, 4.76; N, 5.30; Cl, 6.70%.

3-*Carboxy*-4-*imino*-1-(β-*D*-*ribofuranosyl*)-1, 4-*dihydropyridine* (*clitidine*) **1** *from* **4**

To a soln of **4** (1.3 g) in MeOH (33 ml) and water (12 ml) was added Et_3N (3 ml) and the mixture was allowed to stand at room temp overnight. The solvent was removed under reduced pressure and the residue was applied to a column of charcoal (5 g, packed with water). A 30% aq EtOH eluate afforded crude crystals, which were recrystallized from water to yield **1** (590 mg, 89%). m.p. 190°. $[\alpha]_D^{24°}$ −43.4° (*c* 1.23, H_2O). The IR, UV and ^1H NMR spectra were superimposable on those of the natural product and the chromatographic mobilities (PPC, PEP) were completely identical with those of the natural product.

3-*Carbamoyl*-4-*imino*-1-(β-*D*-*ribofuranosyl*)-1, 4-*dihydropyridine hydrochloride* **5**

(i) *From* **4**. To a soln of **4** (370 mg) in dry MeOH (8 ml) was added satd methanolic ammonia soln (8 ml) and the mixture was kept at room temp overnight. The solvent was removed under reduced pressure and the residue was partitioned with $CHCl_3$ and water. The aqueous layer was evaporated to give a colourless syrup, which was treated with MeOH–n-BuOH (1:10). The resulting precipitate was filtered off, washed with n-BuOH and dried to yield **5** as hygroscopic white powder (201 mg, 94%). $[\alpha]_D^{24°}$ −66.7 (*c* 0.46, H_2O). IR: (nujol) 3400, 3230, 1670, 1595, 1200, 1090, 1065 cm^{-1}, UV; 270 nm (logϵ 4.25, EtOH), EI-MS; *m/e* 137 (base), 120 (base–NH_3), CI-MS; *m/e* 138 (base + H), 133 (sugar), ^1H NMR; (D_2O) 3.93 (2H, bs, 5'-H), 4.31 (3H, bs, 2', 3', 4'-H), 5.74 (1H, d, J = 4.5 Hz, 1'-H), 7.04 (1H, d, J = 7 Hz, 5-H), 8.23 (1H, dd, J = 1.5, 7 Hz, 6-H), 8.78 (1H, d, J = 1.5 Hz, 2-H), PPC; solvent (d) Rf 0.51, PEP; pH 4.6, −3 cm, pH 9.2, −0.5 cm. (Found: C, 42.74; H, 5.55; N, 13.70; Cl. 11.32. Calc. for $C_{11}H_{15}N_3O_5$ HCl: C, 43.21; H, 5.28; N, 13.75; Cl, 11.60%.)

The free state of this compound was identical with an authentic sample obtained from NAD by enzymatic reactions,[27] in chromatographic behavior (PEP, PPC) and spectral properties (^1H NMR IR, UV, $[\alpha]_D$).

(ii) *From* **3**. To a soln of **3** (75 mg, 0.43 mmol) in dry CH_2Cl_2 (1.5 ml) was added 3, 5-di-*O*-benzoyl-D-ribofuranosyl chloride (170 mg, 0.45 mmol) and the mixture was kept at 4° overnight. The solvent was removed under reduced pressure and the residue was dissolved in dry MeOH (1 ml). To this soln was added sat methanolic ammonia soln (1 ml) and the mixture was kept at room temp overnight. The mixture was evaporated to dryness and the residue was partitioned with $CHCl_3$ and water. The aqueous layer was evaporated and the residue was applied to preparative paper chromatography with a solvent system; i-PrOH–water (3:1). A UV active band at R_f 0.4 was cut off, extracted with water and the extracts were evaporated to dryness. The resulting colourless syrup was treated with MeOH–*n*-BuOH (1:10) to yield hygroscopic white powder of **5** (34 mg, 26%). The IR, UV and ^1H NMR were superimposable on those of **5** obtained from **4** and chromatographic mobilities (PPC, PEP) were identical with those of **5** also.

3-*Carboxy*-4-*imino*-1-(2, 3-O-*isopropylidene*-β-*D*-*ribofuranosyl*)-1, 4-*dihydropyridine* (2, 3-O-*isopropylidene clitidine*) **6**

To a suspension of **1** (50 mg) in acetone (5 ml) was added 70% aq $HClO_4$ (0.05 ml) with stirring at 0°, and the mixture was kept for 2.5 h at room temp. The soln was neutralized by the addition of sat $NaHCO_3$ aq. The solvent was removed under reduced pressure and the residue was partitioned with $CHCl_3$ and water. The aqueous layer was evaporated to dryness and the residue was applied to a column of charcoal (0.5 g, packed with water). A 50% aq acetone eluate furnished a crystalline solid, which was recrystallized from water to afford **6** (37 mg, 65%). m.p. 162°. $[\alpha]_D^{24°}$ −112° (*c* 0.5, MeOH). IR (nujol): 3400, 1663, 1583, 1170, 1100, 1080 cm^{-1}. UV: 270 nm (logϵ 4.38, MeOH). EI-MS: *m/e* 310 (M^+), 295 (M^+-CH_3), 252 (M^+-acetone), 157 (sugar-acetonide), 138 (base + H), 120 (base + H-H_2O). Exact Mass: Found: 310.1175, Calc. for $C_{14}H_{18}N_2O_6$ (M^+): 310.1165, ^1H NMR (DMSO): 1.31 (3H, s, exo-Me), 1.56 (3H, s, endo-Me), 3.5–3.8 (2H, m, 5'-H), 4.25–4.45 (1H, m, 4'-H), 4.85 (2H, bs, 2', 3'-H), 5.40 (1H, bs, -OH), 5.95 (1H, s, 1'-H), 6.90 (1H, d, J = 7 Hz, 5-H), 8.24 (1H, dd, J = 1.5, 7 Hz, 6-H), 8.69 (1H, d, J = 1.5 Hz, 2-H).

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