Indole Alkaloids of *Haplophyton crooksii*[1]

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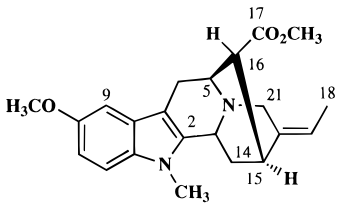
Received November 10, 1995[⊗]

An investigation of the indole alkaloids of *Haplophyton crooksii* has resulted in the identification of two new alkaloids—10-methoxy-*N1*-methylpericyclivine (**1**) and 16-decarbomethoxyvinervinine (**2**)—and 13 known alkaloids—crooksiine, yohimbine, *β*-yohimbine, crooksidine, decarbomethoxytetrahydrosecodine, akuammicine, tubotaiwine, lanceomigine, lanceomigine *N*-oxide, haplophytine, cimicine, cimicidine, and akuammidin—as major alkaloids of this shrub. All of the isolated alkaloids showed an inhibition of acetylcholinesterase activity in vitro.

A number of indole alkaloids are known to possess neurotoxic activity.[3] Mechanistically, these alkaloids may act at the postsynaptic membrane (e.g., curarine C-III and alcuronium), at synaptic ganglia (e.g., garnerine and *β*-yohimbine), or at the junctional sites (e.g., physostigmine and ajmaline).[3] Two species of the genus *Haplophyton* have been reported to possess neurotoxic properties.[4] One species, *Haplophyton cimicidin*, has been extensively investigated, and 12 indole alkaloids were isolated by Cava's group.[5] We previously reported the isolation of crooksiine, yohimbine, *β*-yohimbine,[6] crooksidine, and decarbomethoxytetrahydrosecodine[7] from a second species, *Haplophyton crooksii* L. Benson (Apocynaceae). In addition to the above-mentioned alkaloids we now report that *H. crooksii* contains 10 indole alkaloids, which were isolated and identified as 10-methoxy-*N*1-methylpericyclivine (**1**), 16-decarbomethoxyvinervinine (**2**), akuammicine, tubotaiwine, lanceomigine, lanceomigine *N*-oxide, haplophytine, cimicine, cimicidine, and akuammidine. Of the 15 alkaloids isolated, **1** and **2** are new. All of these alkaloids showed significant in vitro inhibition of acetylcholinesterase.



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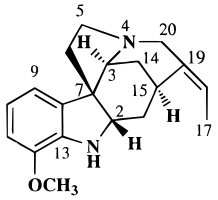

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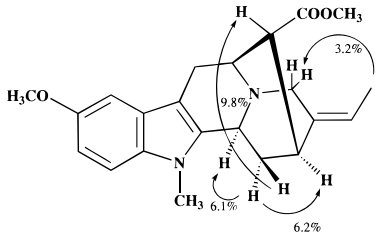
The crude alkaloid fraction was initially separated into five fractions by flash chromatography on celite. The least polar fraction, fraction A (2.04 g, eluted with hexane) upon chromatography on deactivated Si gel, gave five groups of fractions (as determined by TLC), which were processed separately. Further chromatography of fractions followed by HPLC resulted in the isolation of crooksidine,[7] decarbomethoxytetrahydrosecodine,[7] and **1**. The HREIMS of **1** (mp 213−215 °C, $[\alpha]_D$ + 24.06° (*c* 0.32, $CHCl_3$)) established the molecular weight at 366 ($C_{22}H_{26}N_2O_3$). The UV spectrum (λ max 225 and 275 nm) implied the presence of an indole chromophore in **1**.[8] The ^{1}H-NMR spectrum showed the presence of a substituted indole [δ 7.15 (1H, d, J = 8.7 Hz), 6.80 (1H, dd, J = 8.7, 2.4 Hz), 6.78 (1H, d, J = 2.4 Hz)] along with two OMe (δ 3.82 and 3.59), one NMe (δ 3.11, 3H, s), and an ethylidine moiety [δ 5.30 (1H, q, J = 7 Hz) and 1.62 (3H, d, J = 7 Hz)]. The multiplicity of the aromatic protons as well as the connectivities observed in the ^{1}H−^{1}H COSY spectrum suggested the presence of a substituent either on the C-10 or C-11 of the indole nucleus. The ^{13}C-NMR spectrum of **1** was similar to what has been reported for pericyclivine[9] with the exception that **1** had an NMe resonance (δ 53.5), an OMe resonance (δ 56.3), and an upfield shift of one of the indole carbons from δ 117.4 to δ 153.7. Placement of an OMe at C-10 or at C-11 could account for both the additional OMe and an upfield shift of one of the indole carbons in the ^{13}C-NMR spectrum of **1**. On the basis of biogenetic considerations, ^{1}H−^{1}H COSY and HMBC spectra [correlations were observed between H-12 (d 7.15) and C-10 (153.7) and between H-9 (d 6.78), C-13 (133.2), and C-8 (127.1)] the methoxy group was fixed at C-10, and alkaloid **1** was determined to be 10-methoxy-*N*1-methylpericyclivine. The stereochemistries at C3, C15, and C17 were established on the basis of the NOE spectrum (Figure 1).



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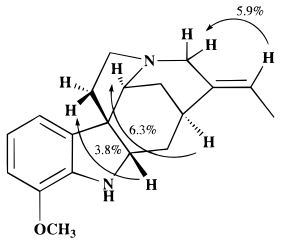

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Figure 1. NOE correlations observed in **1** and **2**.

Fractions 10−19 were combined on the basis of TLC, and an HPLC of the residue resulted in the separation

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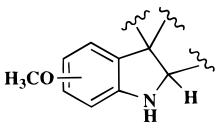
⊗ Abstract published in *Advance ACS Abstracts,* August 1, 1996.

S0163-3864(96)00070-5 CCC: $12.00

of akuammicine and tubotaiwine, which were identified by comparison of their spectra with those in the literature.[9,10] The NOE spectrum was used to differentiate tubotaiwine from (20*S*)-19,20-dihydrocondylocarpine.

Fractions 20−54 contained a single compound (TLC), which, upon combination followed by evaporation, gave 95 mg of a colorless crystalline compound identified as lanceomagine by a comparison of its spectral data with those reported in the literature.[11]

Fractions 126−169 showed the presence of two compounds (TLC) that were separated on a RP2 column to afford the major alkaloid **2** and a minor alkaloid identified as lanceomagine *N*-oxide.[11] The UV spectrum of **2** suggested the presence of a dihydroindole moiety (λ max 228, 259, 300 nm).[8] Both a FABMS and a LREIMS established the molecular ion at *m*/*z* 320 with major fragments at *m*/*z* 174, 160, and 136. The ^{1}H-NMR spectrum also supported the presence of a dihydroindole moiety [6.53 (1H, d, J = 7.6 Hz), 6.71 (1H, d, J = 7.2 Hz), and 6.78 (1H, t, J = 7.4 Hz) in **2**. The number and the multiplicity of the aromatic protons suggested a substituent (OH or OMe) at C-9 or C-12 of the indole moiety. Utilizing a ^{1}H−^{13}C COSY spectrum and on the basis of substitution effects on carbon chemical shifts,[12] a methoxyl was placed on C-12. An ethylidine group was evident from a methine quartet at δ 5.10 and a methyl doublet at δ 1.38. A ^{1}H−^{1}H COSY spectrum suggested fragments **A**, **B**, and **C** in **2** along with an isolated four-spin system represented by protons resonating at δ 3.20, 2.55, 2.02, and 1.70. These correlations suggested that **2** belonged to either type S or type C indole alkaloids represented by vinervinine and pericyclivine, respectively. A HMBC spectrum showed correlations between H-2β (δ 3.30) and C-8 (δ 130.8) and C-13 (δ 139.19), which helped to assign the resonance at 60.0 to C-2. The HMBC spectrum also showed correlations between H-5 and C-3, H-19 and C-3, H-5 and C-7, H-16 and C-19, H-14 and C-19, H-6 and C-8, H-16 and C-19, and OMe and C-12. A pericyclivine-type structure for **2** was eliminated from consideration because of the scalar couplings between H-2 and H-16 (J = 10.7, 6.5 Hz), which are too high to represent a long-range coupling between H-2 and H-16 in a pericyclivine-type structure. Similarly, the observed ^{13}C chemical shift of C-2 (δ 60.0) is considerably lower than what has been reported (between δ 70 and 80) for alkaloids of type C. A comparison of the ^{13}C data of the aliphatic region of **2** with those reported for longicaudatine[13] showed remarkable similarities. On the basis of spectral data, **2** was identified as 16-decarbomethoxyvinervinine. A NOESY spectrum showed a transfer of magnetization between H-18 and H-20, thereby establishing the *E* stereochemistry for the double bond. The spectrum also showed correlations between H-2 and H-16α, H-20α and H-14α, H-15 and H-17, H-3 and H-14, and H-3 and H-16α.



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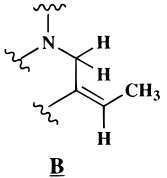

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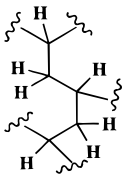

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Table 1. Acetylcholinesterase Inhibition (IC_{50}) of *Haplophton crooksii* Alkaloids and Eserine

alkaloid	IC_{50} ($\times 10^{-4}$ M)
eserine	0.015
β-yohimbine	4.31
crooksidine	1.75
decarbomethoxytetrahydrosecodine	2.03
10-methoxy-*N*1-methylpericycivine (**1**)	1.35
akuammicine	2.21
tubotaiwine	1.08
lanceomagine	3.83
16-decarbomethoxyvinervine (**2**)	0.57
haplophytine	2.25
cimicine	2.41
cimicidine	1.97
akuammidine	1.88

Repeated silica chromatography of fraction B resulted in the separation of two groups of fractions. The less polar group consisted of a mixture of two alkaloids that were separated and identified as cimicine and cimicidine.[14] The more polar group of fractions contained one major alkaloid, which was identified as akuammidine on the basis of its spectral data.[15] The structure was also confirmed by single crystal X-ray crystallography. The crystal data (orthorhombic, $P2_12_12_1$) were similar to what has been reported in the literature for akuammidine.[15]

Flash chromatography of the residue BE resulted in the separation of a group of fractions that were separated by HPLC to afford haplophytine[16] along with cimicidine and cimicine.

All of the isolated alkaloids showed anti-acetylcholinesterase activities at different concentration levels. The most active of the *H. crooksii* alkaloids is about 38 times less active than the known acetylcholinesterase inhibitor eserine ($IC_{95} = 4.8 \times 10^{-6}$ M). The IC_{50} values of each of the isolated compounds is presented in Table 1.

The reported[17] toxic effects of *H. crooksii* could be due to the presence of indole alkaloids. However, the anti-acetylcholinesterase activity may not be the only mode of action accounting for the neurotoxicities of these alkaloids. Although we have not investigated the ganglion-blocking activity of a number of the isolated alkaloids, a comparison of the structures of the isolated alkaloids with known ganlion-blocking agents shows remarkable similarities.[18]

Experimental Section

General Experimental Procedures. NMR spectra were recorded on a General Electric QE 300 spectrometer operating at a frequency of 300.005 and 75 MHz for ^{1}H and ^{13}C nuclei, respectively. ^{1}H−^{1}H COSY and ^{1}H−^{13}C COSY, HMBC, and NOE spectra were recorded according to standard pulse sequence.[19] HPLC analysis was carried out on a Waters HPLC, Model LC 1200, which was equipped with a Waters Model 440 UV detector, Fisher Recordall Series 5000 recorder, Si 10 μm Radial-Pak cartridge (8 mm × 10 cm) in a radial compression module (RCM 100). TLC was carried out on Si gel 60 plates (E. Merck). Ceric ammonium sulfate was used as the visualization agent. Melting points were recorded on a Fisher-Johns apparatus and were uncorrected; LREIMS were run on a Finnigan Model 1020 spectrometer equipped with an Incos data system; HRMS, FABMS, and HRFABMS were recorded at the

Midwest Center for Mass Spectrometry, University of Nebraska; IR spectra were recorded on a Perkin-Elmer 283 spectrophotometer; UV spectra were obtained on a Perkin-Elmer 200 spectrophotometer. A Beckman 26 spectrophotometer was used for recording the OD in the determination of acetylcholinesterase activity.

Plant Material. *Haplophyton crooksii* was collected from Franklin Mountain, El Paso, Texas, in September 1989, and was identified by Dr. C. Freeman, Department of Biology, University of Texas−El Paso. A voucher specimen is deposited at the Department of Pharmacological and Pharmaceutical Sciences, College of Pharmacy, University of Houston.

Extraction and Isolation of Alkaloids. Plant material (aerial parts 3.50 kg, roots 0.55 kg) was air-dried, ground, and exhaustively extracted with MeOH. Each part of the plant was processed separately; roots were extracted (1.5 L MeOH) using a Soxhlet apparatus, while the aerial parts were allowed to stand in MeOH (5 L) at room temperature for one week. The MeOH extracts were filtered and concentrated under reduced pressure to afford 51.7 g (brown resin) and 188.2 g (green resin) of residues from roots and aerial parts, respectively. Each residue was suspended in 750 mL of 0.1% HCl and extracted with 2 L (3 ×) of $CHCl_3$. The pH of the aqueous layer was adjusted to pH 11 with NH_4OH followed by extraction with $CHCl_3$. The second organic layer was washed with distilled H_2O, dried over anhydrous Na_2SO_4, and evaporated to dryness to afford 3.84 g (6.9 g/kg) and 1.86 g (0.53 g/kg) of crude alkaloids from the roots and aerial parts, respectively. Both alkaloidal fractions were combined, because they exhibited similar constituents on TLC [Si gel; MeOH−$CHCl_3$−NH_4OH (10:90:0.5 v/v)], to give the crude alkaloid residue.

The marc from the aerial parts, after cold MeOH extraction, was reextracted with basic MeOH (pH 10, NH_4OH). The MeOH extract was neutralized (pH 7) with 0.5% HCl and concentrated to afford 3.80 g of crude alkaloids.

The alkaloidal residue was subjected to flash chromatography on celite (35 × 5.5 cm; 240 g of celite) for preliminary fractionation. The column was eluted with hexanes (6 L), hexanes−$CHCl_3$ (50:50 v/v, 7 L), $CHCl_3$ (5 L), 50% $CHCl_3$−MeOH (50:50 v/v, 4 L), and MeOH (2 L). Upon evaporation, the eluents afforded residues A−E (A, 2.04 g; B, 2.94 g; C, 0.27 g; D, 0.21 g, and E, 0.17 g) from hexanes, 50% hexanes−$CHCl_3$, $CHCl_3$; 50% $CHCl_3$−MeOH, and MeOH, respectively.

Secodisiine and Decarbomethoxytetrahydrosecodine. Repeated column chromatography of residue A on deactivated Si gel followed by alumina chromatography of the desired fractions resulted in the separation of a group of fractions that, upon HPLC, using two Si Radial-Pak cartridges and MeOH−$CHCl_3$−NH_4OH (1.25:98.75:0.1 v/v) as the mobile phase, resulted in the separation of 8.3 mg of decarbomethoxytetrahydrosecodine and 5.6 mg of secodisiine.

10-Methoxy-*N*1-methylpericyclivine (1). A deactivated Si gel column chromatography (230−400 mesh, column 35 × 0.75 cm) of the residue from the least polar fraction of first column chromatography [MeOH−$CHCl_3$−NH_4OH (2:98:0.7 v/v) as the solvent system] gave a group of fractions that, upon HPLC [MeOH−$CHCl_3$−NH_4OH (0.75:99.25:0.075 v/v) as the mobile phase], resulted in the separation of 7.2 mg of **1**: mp 213−215 °C, $[\alpha]_D$ 24.06 (*c* 0.32, $CHCl_3$), UV λ max ($CHCl_3$) 285 (log ϵ 4.15), 242 (log ϵ 4.30); IR ν max ($CHCl_3$) 3200, 2910, 1720, 1450, 1280, 1150, 1020 cm^{-1}; HRMS *m/z* (% rel int) 366.14559 (100) [calcd for $C_{22}H_{26}N_2O_3$, 366.14633], 365.1882 (77), 351.1692 (22), 307.1813 (34), 293.1657 (14), 213.1015 (54), 212.0952 (93), 197.0725 (13); ^{1}H NMR δ 1.62 (d, J = 7.0 Hz, 18-CH_3), 1.75 (1H, m, H-14β), 2.51 (1H, m, H-14α), 2.63 (1H, m, H-5α), 2.83 (1H, dd, J = 10.2, 2.3 Hz, H-16), 2.92 (1H, m, H-6β), 3.00 (1H, m, H-15β), 3.11 (3H, s, N-Me), 3.20 (1H, H-6α), 3.59 (3H, s, O-Me), 3.63 (2H, m, H-21), 3.82 (3H, s, O-Me), 4.30 (1H, d, J = 10 Hz, H-3α), 5.30 (1H, q, J = 7 Hz, H-19), 6.78 (1H, d, J = 2.4 Hz, H-9), 6.80 (1H, dd, J = 8.7, 2.4 Hz, H-11), 7.15 (1H, d, J = 8.7 Hz, H-12); ^{13}C NMR 12.8 (C-18), 24.1 (C-14), 26.2 (C-6), 27.0 (C-15), 37.9 (N-Me), 43.75 (C-3), 49.6 (C-16), 50.9 (O-Me), 53.0 (C-21), 56.0 (C-5), 56.3 (O-Me), 100.4 (C-9), 103.83 (C-7), 109.4 (C-12), 110.3 (C-11), 114.7 (C-19), 127.1 (C-8), 133.2 (C-13), 138.8 (C-20), 139.2 (C-2), 153.7 (C-10), 172.7 (C-17).

Akuammicine and Tubotaiwine. Repeated HPLC [Si, MeOH−$CHCl_3$−NH_4OH (2.5:97.5:0.75 v/v) as the mobile phase] of the residue from the second group of fractions from the first deactivated Si gel chromatography resulted in the separation of two alkaloids, akuammicine (4.82 mg) and tubotaiwine (10.78 mg).

Lanceomigine. Isolated as a pure compound from the second column chromatography, this compound was identified as lanceomigine on the basis of its spectral data.

16-Decarbomethoxyvinervinine (2). Residue from fractions of medium polarity upon RP2 column chromatography [30 × 0.5 cm, MeOH−$CHCl_3$−$(C_2H_5)_3N$ (5:95:0.5 v/v)] yielded 22 fractions (3 mL each), which were combined on the basis of TLC. Fractions 5−8 gave a single spot on TLC (RP2, MeOH−$CHCl_3$−$(C_2H_5)_3N$ (10:95:0.5 v/v), which was identified as lanceomigine. Combined fractions 11−18 upon evaporation afforded 10.2 mg of a glassy residue (**2**): UV λ max (EtOH) 285 (log ϵ 3.6), 243 (log ϵ 4.06), and 208 (log ϵ 4.70) nm; FABMS *m/z* (% rel int) 321 (M^+ + H, 100); eims *m/z* (70 eV, % rel int) 320 (M^+, 56), 174 (32), 160 (73), 136 (100); ^{1}H NMR δ 0.92 (1H, dt, J = 13.5, 3.0 Hz, H-16β), 1.70 (2H, m, H-16a, H-6β), 1.31 (1H, d, J = 13.6 Hz, H-14β) 1.38 (3H, d, J = 6.9 Hz, H-17), 1.90 (1H, dd, J = 15.8, 2.1 Hz, H-14α), 2.02 (1H, dd, J = 12.4, 6.5 Hz, H-6α), 2.28 (1H, m, H-15α), 2.55 (1H, m, H-5β), 2.60 (1H, d, J = 15.4 Hz, H-20β), 2.95 (3H, s, OCH_3), 3.20 (1H, J = 9.2 Hz, H-5α), 3.30 (1H, m, H-2β), 3.70 (1H, d, J = 15.4 Hz, H-21α), 3.92 (1H, bs, H-3α), 5.10 (1H, q, J = 7.3 Hz, H-18), 6.53 (1H, d, J = 7.3 Hz, H-11), 6.71 (1H, d, J = 7.2 Hz, H-9), 6.78 (1H, t, J = 7.2 Hz, H-10); ^{13}C NMR 13.0 (C-17), 25.4 (C-14), 27.6 (C-15), 35.3 (C-16), 37.9 (C-6), 38.6 (N-Me), 51.9 (C-5), 53.8 (C-7), 54.0 (O-Me), 54.6 (C-20), 59.6 (C-3), 60.0 (C-2), 110.0 (C-11), 114.8 (C-9), 119.8 (C-10), 123.0 (C-18), 130.8 (C-8), 134.2 (C-19), 139.1 (C-13), 145.9 (C-12).

Lanceomigine *N*4-Oxide. Si gel column chromatography of the residue from polar fractions followed by HPLC [two Radial-PAK Si cartridges, MeOH−$CHCl_3$−NH_4OH (5:95:0.4 v/v)] of the desired fractions resulted in the separation of lanceomigine *N*4-oxide.

Residue B (1 g), upon deactivated Si gel column chromatography utilizing MeOH−$CHCl_3$−NH_4OH (2.5:

97.5:0.25 v/v) as the eluting solvent, resulted in the separation of six combined fractions [fractions 1−14, 0.23 g; 15−28, 0.18 g; 29−53, 0.34 g; 54−69, 0.09 g; and 70−100, 0.11 g].

Cimicidine and Cimicine. Repeated Si gel column chromatography of the residue from the combined fraction 15−28 resulted in the separation of two groups of fractions that were combined on the basis of TLC. The residue from the first of these, fractions 18−21, upon fractional crystallization resulted in the separation of cimicidine and cimicine.

Akuammidine. Attempts to dissolve the residue (30.63 mg) from the second group, fractions 22−35, in cold $CHCl_3$ resulted in the separation of a white precipitate that was crystallized in EtOH to afford 30.2 mg of colorless needles, which were identified as akuammidine on the basis of its spectral data.

Haplophytine. Repeated HPLC (Si, 57 cm, 1 cm; 1.5 mL/min) utilizing MeOH−$CHCl_3$−NH_4OH (2.0:98.0:0.2 v/v) as the mobile phase afforded 9.5 mg of pure alkaloid haplophytine from the alkaloid fraction obtained from the alkaline MeOH extraction of the marc.

In Vitro Acetylcholinesterase Assay. In vitro inhibition of acetylcholinesterase activity by the isolated compounds was determined by a slight modification of the method developed by Rappaport *et al*.[20] Eserine was used as a standard. Acetylcholinesterase from electric eel (500 units) was dissolved in 10 mL of 0.1 M KH_2PO_4 buffer (pH 7.0) and kept frozen. Working solutions were prepared on the day of the experiment by reconstituting each vial (150 mg) with 4 mL of distilled H_2O. The color indicator solution was prepared by dissolving 0.75 g of *m*-nitrophenol in 1 L of 0.1 M phosphate buffer (pH 7.8) and was refrigerated. Solutions of alkaloids were prepared in EtOH and stored at below-freezing temperatures. In a typical experiment, a mixture consisting of 100 μL (5.0 units) of acetylcholinesterase, 50 μL of substrate (acetylcholine), 150 μL of *m*-nitrophenol solution, different concentrations of the alkaloids, and 3 mL of distilled H_2O was incubated for exactly 30 min at 25 °C. After the incubation period the absorbance of the solution was determined at 412 nm. The control experiment involved incubating the enzyme (100 μL), substrate (50 μL), *m*-nitrophenol (150 μL), and 3 mL of distilled H_2O. The percent inhibition (%*I*) of the enzyme was calculated according to the formula $\%I = (DA \times 100)/DA_0$, where $DA = A_n - A_0$; A_n = absorbance at different concentrations of samples, A_0 = absorbance at zero inhibition (i.e., enzyme is active) and $DA_0 = A_f - A_0$; A_f = absorbance at 100% inhibition (i.e., denatured enzyme).

Acknowledgment. This work was supported, in part, by a grant from the University of Houston Coastal Center. Technical assistance of Ms. Ziva Yousefipour, in the determination of anti-acetylcholinesterase activity, is also acknowledged.

References and Notes

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NP960070C