Cytotoxic Degradation Product of Physostigmine

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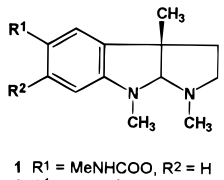
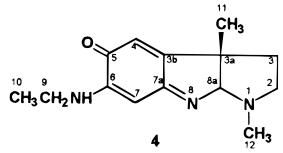
Rubreserine (**3**), a known degradation product of physostigmine (**1**), and a new indole derivative, **4**, were isolated when **1** was refluxed with NH_4OH. The structure of the rearrangement product of **1,** compound **4,** was deduced through the interpretation of NMR data. Rubreserine (**3**) was very weakly cytotoxic to KB (human oral epidermoid carcinoma) and LNCaP (hormone-dependent human prostate cancer) cells and was active in the ASK (astrocytoma) assay. Compound **4** showed potent cytotoxicity in all cell lines tested.

Physostigmine (eserine) (**1**), an indole alkaloid isolated from the Calabar bean (*Physostigma venenosum* Balf., Leguminosae), is well known as a clinically useful inhibitor of acetylcholinesterase.[1] Ethnopharmacologically, the plant was used in the southeast region of Nigeria in rituals and as an ordeal poison, which resulted in paralysis of the lower limbs and death by asphyxia and, in larger doses, paralysis of the heart.[2,3] Colored decomposition products of physostigmine were observed during the attempts to purify this alkaloid from the Calabar bean,[4−6] and the mechanism of the decomposition was first studied by Ehrenberg,[4] and later by Salway.[7] The primary reaction in the decomposition of physostigmine is the hydrolysis of the methylcarbamyl side chain to afford a colorless phenolic compound, eseroline (**2**).[4,7] Eseroline is then oxidized to the red *ortho*-quinone rubreserine (**3**).[8] Further degradative reactions result in the formation of eserine blue and eserine brown, whose structures have remained elusive. The anticholinesterase activities of these four degradation products of physostigmine have been determined in vitro using biochemical and biological assays and in vivo using a chromodacryorrhea test in rats, and they were all at least 100 times less active than physostigmine in both assays.[9] The current report describes the isolation, the structure elucidation, and the cytotoxicity of **4**, a degradation product when physostigmine in absolute EtOH is refluxed with NH_4OH on a steam bath.



1 R^1 = MeNHCOO, R^2 = H
2 R^1 = OH, R^2 = H
3 R^1 = R^2 = O



4

Rubreserine (**3**) was isolated from the reaction mixture and identified by comparison of its ^{1}H-NMR data with those of an authentic sample, which was prepared for this study from a separate reaction according to the method of Robinson.[10] During the course of structure confirmation, the ^{1}H-NMR assignments were revised from those proposed by Robinson.[10] The resonances of the protons at δ 3.02 (H-2A), δ 2.89 (H-2B), and δ 1.99 (H-3A and H-3B) on two methylene carbons appeared as doublets of triplets with coupling constants of 2.6 and 6.4 Hz. The ^{13}C-NMR data for **3** are reported here for the first time. The carbon chemical shifts at δ 182.6 and at δ 174.8, indicating the presence of two carbonyl carbons, supported the structure of **3** originally proposed by Coyne and Paterson.[11] Irradiation of H-7 and H-4 ($^3J_{CH}$ = 7 Hz) in a selective INEPT experiment enhanced the resonances of C-5 and C-6, respectively, confirming the location of these two carbonyl carbons.

Compound **4** appeared as a red spot on TLC after spraying with Dragendorff's reagent, the same color as **3,** but with a higher R_f value. The IR spectrum suggested the presence of imide (ν max 3400 cm^{-1}), imine (ν max 1653 cm^{-1}), and carbonyl (ν max 1601 cm^{-1}) groups. HREIMS gave a molecular weight of 245.1539 amu, corresponding to a molecular formula of $C_{14}H_{19}N_3O$. A small (11%) M − 1 peak (*m*/*z* 244) was detected below the molecular ion peak (*m*/*z* 245) (100%). This loss of a hydrogen radical is a characteristic feature of those alkaloids with a tetrahydropyrrolo[2,3-*b*]indole system,[12] because the loss of the aminal proton generates an immonium ion in which the positive charge may be delocalized between the two nitrogens. Loss of the quaternary methyl radical from **4** furnished an ion at *m*/*z* 230 (18%), and loss of the CH_3CH_2NH side chain led to an ion at *m*/*z* 201 (48%).

The ^{1}H-NMR spectrum of **4** showed a methyl singlet at δ 1.42, resembling the chemical shift of the methyl signal on C-3a of **1**, and a methyl triplet at δ 1.29, indicating the presence of an ethyl group in the molecule. Only one *N*-methyl singlet at δ 2.68 was observed, compared with the three *N*-methyl groups, which appeared in **1** as two singlets at δ 2.54 (N-1) and at δ 2.91 (N-8), and a doublet at δ 2.87 (N-10). These data suggested that compound **4** had two *N*-methyl groups fewer than **1**. The proton singlet at δ 4.87, shifted downfield from δ 4.11 in **1**, was assigned to a methine at C-8a. The N-H signal was also moved downfield, from δ 4.93 in **1** to δ 5.35 in **4**, implying a deshielding environment. Critically, there were only two proton singlets in the aromatic region instead of the three resonances that appeared in **1**, indicating the presence of two *para*-related protons on the aromatic ring (i.e., on C-4 and C-7). The chemical shift of H-4 (δ 6.22) was quite similar to that of H-4 (δ 6.24) in **3**.

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Table 1. DQCOSY Correlations and Selective INEPT Enhancements of Compound **4**

position	DQCOSY	selective INEPT ($^3J_{CH}$ = 6 Hz)
2A	2B, 3A, 3B	3a, 8a
2B	2A, 3A, 3B	12
3A	2A, 2B, 3B	(2, 3, 3a),[a] 3b, 11
3B	2A, 2B, 3A	(3a),[a] 3b, 8a, 11
4		6, (7),[a] 7a
7		3b, 5
8a		2, 7a
9	10	6, 10
N-H		(5, 7)[b]

[a] Entries represent non-3-bond enhancements. [b] $^3J_{CH}$ = 8 Hz.

However, the chemical shift of H-7 in **4** was more downfield (δ 5.93) than that of H-7 (δ 5.35) in **3**. The ^{13}C-NMR showed only one carbonyl carbon at δ 183.2, which was close to that of C-5 (δ 182.6) in **3**, and, according to the degradation pathway of **1**, this carbonyl carbon of **4** was assigned to C-5. Thus, the presence of a substituent group on C-6 in **4** was deduced. From DQCOSY (Table 1), a strong correlation between the proton at δ 5.35 (N-H), and the protons at δ 3.16 (on C-9) was observed, which indicated that the ethyl group was adjacent to the NH group. In the selective INEPT experiment (Table 1), irradiation of the proton (δ 5.35, $^3J_{CH}$ = 8 Hz) on this NH group enhanced the resonances of both the carbonyl carbon at δ 183.2 (C-5), and the methine carbon at δ 93.3 (C-7), thereby confirming the assignments of C-5 and C-7, respectively. This experiment also established that the CH_3CH_2NH- group was indeed the substituent at C-6 of the aromatic ring. Additionally, selective enhancements of C-6 (δ 144.6) and C-7a (δ 166.1) were observed when the protons at δ 3.16 (on C-9), and at δ 4.87 (on C-8a) were irradiated ($^3J_{CH}$ = 6 Hz), respectively. The unusual mechanistic formation of **4** is presently under investigation.

Rubreserine and compound **4** were evaluated for their cytotoxic potential using a battery of human cancer cell lines (Table 2). Rubreserine was found to be active in the ASK assay (at 100 μg/mL), and only very weakly active in the KB-V (−VLB) (ED_{50} 16.8 μg/mL) and in the LNCaP (ED_{50} 13.1 μg/mL). On the other hand, compound **4** was very active in all of the human cancer cell lines tested (ED_{50} < 4 μg/mL), but not in the ASK assay. The mechanism of this cytotoxic response, which is presently under investigation, may resemble that of compounds in the 9-hydroxyellipticine series.[13]

Experimental Section

General Experimental Procedures. Physostigmine was purchased from Sigma Chemical Co. Column chromatography utilized Si gel 60H (Merck, 0.01−0.04 mm); and TLC, Si gel (Merck). TLCs were viewed under a UV lamp (Chromato-Vue® C-70 G UV Viewing System) and by spraying with Dragendorff's reagent. Melting point was obtained from Fisher-Johns Melting apparatus and is uncorrected. CD spectra were measured on a JASCO J-710 CD/ORD spectropolarimeter. UV spectra were obtained in MeOH, using a Beckman DU-7 spectrometer, and IR spectra were obtained on a Midac Collegian FT-IR spectrometer. The NMR spectra were recorded on a Varian XL-300 NMR spectrometer at 299.9 MHz (^{1}H) and at 75.4 MHz (^{13}C, APT) in $CDCl_3$, using tetramethylsilane (TMS) as an internal standard. DQCOSY and HETCOR were also obtained using the Varian XL-300 NMR spectrometer standard programs. DEPT and selective INEPT were performed on a Nicolet NMC-360 NMR spectrometer (360 MHz for ^{1}H, and 90.8 MHz for ^{13}C). LRMS and HRMS were obtained from a Finnigan MAT 90 mass spectrometer operating at 70 eV.

Degradation Reaction. The degradation reaction was modified from the preparation of eserine blue from physostigmine as described by Ellis.[14] Physostigmine (200 mg) was dissolved in absolute EtOH (1 mL), and NH_4OH (30%) (0.13 mL or 4 drops) was added. The reaction mixture was refluxed on the steam bath for 2 h or until a very small amount of physostigmine was detected by TLC, using cyclohexane−EtOAc−diethylamine (5:2:0.5) as developing solvent, and spraying with Dragendorff's reagent.

Extraction and Isolation of Compounds 3 and 4. The degradation products were extracted from the reaction mixture into $CHCl_3$. Anhydrous $(Na)_2SO_4$ was added into the $CHCl_3$ fraction, and the filtered and evaporated $CHCl_3$ fraction was then dried under vacuum. Elution of the $CHCl_3$ fraction (122.3 mg) with cyclohexane−EtOAc−diethylamine (5:1.5:0.5) from a Si gel 60H column yielded fractions 10−40, which, when combined (69.6 mg), contained physostigmine (**1**) (R_f 0.38), rubreserine (**3**) (R_f 0.28), and **4** (R_f 0.45). Further purification of this combined fraction by preparative TLC, using the same solvent system, gave rubreserine (**3**) as a red solid (3.6 mg) and **4** as a yellow-orange solid (4.1 mg).

Rubreserine (3): a red solid from cyclohexane−EtOAc−diethylamine and red needles after recrystallization from $CHCl_3$/petroleum ether; mp 98−99 °C; CD $[\theta]_{319}$ +6361, $[\theta]_{376}$ −2299; UV (MeOH) λ max (log ϵ) 295 (4.05), 473 (3.64) nm; IR (film) ν max 3035, 2926, 2859, 2357, 1600 (C=O, carbonyl), 1494, 1451, 756, 710 cm^{-1}; ^{1}H NMR ($CDCl_3$, 299.9 MHz) δ 6.24 (1H, s, H-4), 5.35 (1H, s, H-7), 4.36 (1H, s, H-8a), 3.08 (3H, s, H-10), 3.02 (1H, dt, J = 2.6, 6.4 Hz, H-2A), 2.89 (1H, dt, J = 2.6, 6.4 Hz, H-2B), 2.68 (3H, s, H-11), 1.99 (2H, dt, J = 2.6, 6.4 Hz, H-3A and H-3B); ^{13}C NMR ($CDCl_3$, 75.4 MHz) δ 182.6 (s, C-5), 174.8 (s, C-6), 160.0 (s, C-3b), 157.6 (s, C-7a), 123.1 (d, C-4), 96.9 (d, C-8a), 91.9 (d, C-7), 54.6 (t, C-2), 50.5 (s, C-3a), 40.8 (q, C-10), 39.3 (t, C-3), 32.2 (q, C-11), 23.1 (q, C-9); EIMS (70 eV) m/z [M]$^+$ 232 (91), 205 (14), 204 (88), 203 (23), 190 (37), 177 (32), 176 (100).

Compound 4: a yellow-orange solid from cyclohexane−EtOAc−diethylamine; CD $[\theta]_{215}$ +16043, $[\theta]_{241}$ −10 282, $[\theta]_{290}$ +4690; UV (MeOH) λ max (log ϵ) 227

Table 2. Evaluation of Cytotoxic Activities of Rubreserine (**3**) and Compound **4**

	cell lines tested[a] (ED_{50}, μg/mL)						
compound	Lu-1	KB	KB-V (+VLB)	KB-V (−VLB)	LNCaP	ZR-75-1	ASK
rubreserine	>20	>20	>20	16.8	13.1	>20	+
compound **4**	1.5	0.2	0.6	0.8	0.3	1.9	−

[a] Lu-1 = human lung cancer; KB = human oral epidermoid carcinoma; KB-V (+VLB) = vinblastine-resistant KB with 1 μg/mL vinblastine; KB-V (−VLB) = vinblastine-resistant KB without vinblastine; LNCaP = hormone-dependent human prostate cancer; ZR-75-1 = hormone-dependent breast cancer; ASK = astrocytoma (indicates an antimitotic response).

(3.76), 295 (sh, 3.41), 398 (sh, 3.10) nm; UV (MeOH/HCl) λ max (log ϵ) 231 (3.76), 303 (3.47), 372 (sh, 3.24), 398 (sh, 3.21) nm; UV (MeOH/NaOH) λ max (log ϵ) 223 (3.99), 246 (sh, 3.66), 295 (sh, 3.50), 398 (sh, 3.16) nm; IR (film) ν max 3400 (NH), 2976, 1653 (C=N), 1601 (C=O, carbonyl), 1489, 1399, 837 cm^{-1}; ^1H NMR ($CDCl_3$, 299.9 MHz) δ 6.22 (1H, s, H-4), 5.93 (1H, s, H-7), 5.35 (1H, s, N-H), 4.87 (1H, s, H-8a), 3.16 (2H, dq, J = 1.8, 7.3 Hz, H-9), 2.77 (1H, ddd, J = 2.9, 6.8, 9.5 Hz, H-2A), 2.68 (3H, s, H-12), 2.41 (1H, dt, J = 5.8, 9.5 Hz, H-2B), 1.95 (1H, ddd, J = 6.8, 9.5, 12.2 Hz, H-3A), 1.76 (1H, ddd, J = 2.9, 5.8, 12.2 Hz, H-3B), 1.42 (3H, s, CH_3-3a), 1.29 (3H, t, J = 7.3 Hz, H-10); ^{13}C NMR ($CDCl_3$, 75.4 MHz) δ 183.1 (s, C-5), 166.1 (s, C-7a), 162.3 (s, C-3b), 144.6 (s, C-6), 119.0 (d, C-4), 101.6 (s, C-8a), 93.3 (d, C-7), 52.6 (t, C-2), 51.6 (s, C-3a), 39.9 (t, C-3), 37.6 (q, C-12), 37.1 (t, C-9), 23.6 (q, C-11), 13.7 (q, C-10); HREIMS (70 eV) m/z 245.1539 ($C_{14}H_{19}N_3O$ requires 245.1528); EIMS (70 eV) m/z $[M]^+$ 245 (100), 244 (11), 230 (18), 216 (21), 203 (95), 202 (49), 201 (48), 187 (58).

Evaluation of Cytotoxic Activity. Compounds **3** and **4** were evaluated for their cytotoxic activity profile as shown in Table 2, using procedures described previously.[15,16]

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