Blue Degradation Products of Rubreserine

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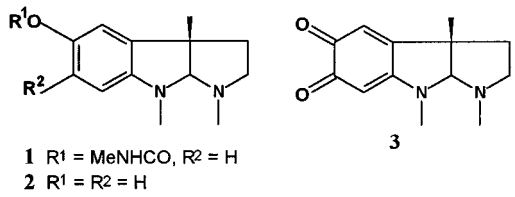
The red, *o*-quinone degradation product of physostigmine (**1**), rubreserine (**3**), was refluxed in absolute EtOH under NH_3 gas to afford two blue, isomeric products, compounds **5** and **6**. The structures of these two blue compounds were derived from the consideration of their spectral data.

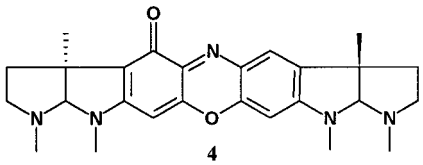
The colored degradation products of physostigmine (**1**) (eserine), an indole alkaloid from the Calabar bean (*Physostigma venenosum* Balf., Leguminosae), and its decomposition mechanism have been studied previously under various conditions.[1−6] Jobst and Hesse in 1864,[1] and Hesse in 1867[2] observed a red color in the alkaline solutions of **1**, which appeared slowly in $NaHCO_3$ solution, more rapidly in Na_2CO_3 solution, and immediately in NH_4OH solution. Ellis *et al.*[7] also found that the destruction of **1** in phosphate buffer solutions (pH 5−8) depended on the hydroxide ion concentrations. These alkaline conditions cause the hydrolysis of the carbamate side chain of **1** to afford a phenolic, colorless compound, eseroline (**2**). In the presence of alkali, compound **2** absorbs oxygen rapidly and is then oxidized to rubreserine (red) (**3**), eserine blue (blue), and eserine brown (brown).[3,8] The structure of **3** was confirmed as a resonance hybrid of the mesomers (between *o*-quinone and quinone methide) by Robinson[9,10] from UV, IR, and ^{1}H-NMR data in 1965, and by Schönenberger *et al.*[11] in 1986 from X-ray diffraction analysis. The empirical formula of eserine blue was suggested as $C_{17}H_{23}N_3O_2$ by Salway[3] in 1912, and in 1967, Auterhoff and Hamacher[6] proposed the structure of eserine blue as **4**, a dimer of **3** with the molecular formula of $C_{26}H_{31}N_5O_3$. Aspects of the chemistry of **1** have been reviewed.[12,13]

Recently, we reported on the isolation and the cytotoxic activity of a new, rearranged degradation product (red-orange)[14] that is formed when **1** in absolute EtOH was refluxed with NH_4OH. In this report, we have modified the preparation of eserine blue from **1**, as described by Ellis,[8] in order to study the degradation of **3** under NH_3 gas, and this has resulted in the isolation and structure elucidation of two blue isomers, compounds **5** and **6**.



1 R^1 = MeNHCO, R^2 = H
2 R^1 = R^2 = H

3



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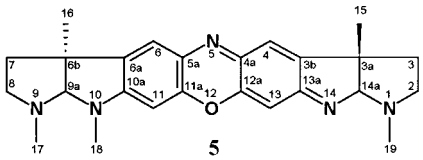
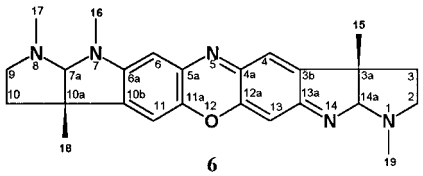


5



6

Results and Discussion

Compound **3** was prepared for this study according to the method of Robinson.[9] The EtOH solution of **3** was refluxed under NH_3 gas for 4.5 h, and the reaction was stopped before the blue color developed and turned brown. The reaction mixture was then subjected to an Al_2O_3 (neutral) column using $CHCl_3$ as an eluent to yield fractions 23−29 and fractions 30−32, respectively. Fractions 23−29 were combined and purified, using an HPLC with an RP-18 column and 0.1% HOAc in DI H_2O−CH_3CN (5:95) as a mobile phase, to give the isomer **5** (3.5 mg) at 4.4 min as a blue compound. Fractions 30−32 from the Al_2O_3 column were pure, and they were combined to give the isomer **6** (5.3 mg), also as a blue compound. The low yield of these two compounds indicated that these are transition products in that the reaction was not complete, and the products can degrade further to form eserine brown inasmuch as the reaction mixture turned darker when allowed to proceed.

The isomers **5** and **6** appeared as blue spots on neutral Al_2O_3 TLC with the same R_f values and the same masses (i.e., 415.2452 amu for **5** and 415.2456 amu for **6**), which correspond to a molecular formula of $C_{25}H_{29}N_5O$. They also showed similar UV, IR, and ^{1}H- and ^{13}C-NMR spectra. The IR spectrum showed only an imine (νmax 1625 cm^{-1}) functionality, the carbonyl groups of rubreserine being absent. Slight differences

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Table 1. COSY Correlations of Compounds **5** and **6**

compd **5**		compd **6**	
^{1}H	correlations	^{1}H	correlations
2α and 2β	3α, 3β	2α	2α, 3α, 3β
3α	2β, 3β	2β	2α, 3β
3β	2α, 2β, 3α	3α	2α, 3β
7α	7β, 8α	3β	2α, 2β, 3β
7β	7α, 8α, 8β	9α and 9β	10α and 10β
8α	7α, 7β, 8β	10α and 10β	9α and 9β
8β	7β, 8α		

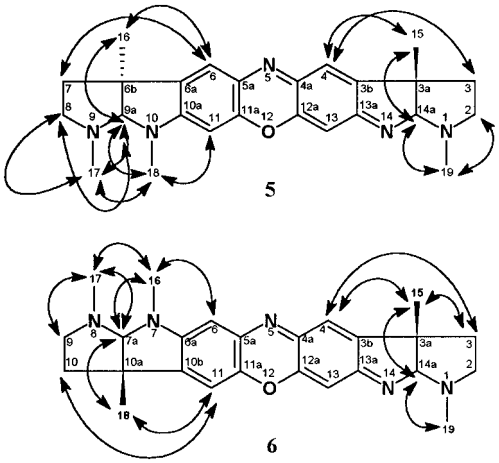

Figure 1. NOESY correlations of compounds **5** and **6**.

in the aromatic ^{1}H-NMR chemical shifts (i.e., δ 7.18, 6.89, 6.79, and 6.16 for **5**, and δ 7.10, 6.70, 6.60, and 6.08 for **6)** established that these two compounds were isomers. The chemical shifts of eight methylene protons (δ 1.86, 2.01, 2.49, 2.80, 1.98, 2.03, 2.75, and 2.75 for **5**, and δ 1.81, 1.96, 2.48, 2.75, 2.71, 2.71, 1.99, and 1.99 for **6**), three *N*-methyl (δ 3.08, 2.65, and 2.60 for **5**, and δ 3.02, 2.64, and 2.57 for **6**), and two methyl groups (δ 1.47 and 1.45 for **5**, and δ 1.43 and 1.41 for **6**) were very similar to those of **3**, indicating that the pyrrolidine portions of **3** had remained intact (with the exception of one *N*-methyl group). The masses and the proton numbers of **5** and **6** were twice those of **3**, implying that these two isolates were dimers of a derivative of **3**. The molecular formula, with only one oxygen and five nitrogen atoms, suggested that the *o*-quinone groups of two molecules of **3** had reacted with NH_3 to form a phenoxazine ring, linking the two molecules together. There were two possibilities in the formation of **5** and **6** from two molecules of the derivative of **3**, that is, the two monomeric units were in the same orientation or in the opposite orientation (all of the pyrrolopyrrolidine nitrogens were on the same side). The other two possible isomers of **5** and **6**, where the oxygen and nitrogen were at positions 5 and 12, respectively, were ruled out because these latter two isomers did not provide a conjugated system in the central portion of these two molecules as in the two proposed isomers.

The COSY correlations of **5** and **6** (Table 1) confirmed the positions of eight methylene protons in these two compounds, and the HMQC correlations supported the assignments of all of the carbons of the methine, methylene, and methyl groups. The NOESY (Figure 1) correlations between the C-15 methyl group and H-4, the C-16 methyl group and H-6, and the methyl group on N-10 and H-11 of **5**, and between the C-15 methyl group and H-4, the methyl group on N-7 and H-6, and the C-18 methyl group and H-11 of **6** supported the

Table 2. HMBC Correlations of Compounds **5** and **6** ($^3J_{CH}$ = 7 Hz)

compd **5**		compd **6**	
^{1}H	^{13}C	^{1}H	^{13}C
2α and 2β	3a, 14a, 19	6	11a
3α	(2, 3a),[a] 3b, 14a, 15	7a	9, 17
3β	(2, 3a),[a] 3b, 14a, 15	9α and 9β	7a, 10a, 10[a]
6	11a	10α and 10β	(9, 10a)[a], 10b
7α	(6b, 8)[a]	13	4a, (13a)[a]
7β	6a	14a	2, 13a, 19
14a	13a	15	2[a]
15	3, (3a),[a] 3b, (13a),[a] 14a	16	7a, (10, 10a, 10b)[a]
16	6a, (6b, 10a)[a]	17	7a, 9
17	8, 9a	18	7a, (10a)[a]
19	2, 14a	19	2

[a] Carbon numbers in parentheses indicate non-three-bond enhancements.

assignments of H-4, H-6, and H-11 of **5** and **6**, respectively. As described previously, the COSY, HMQC, and NOESY correlations allowed the confident assignments of all of the proton and carbon resonances in the pyrrolopyrrolidine portions of both compounds.

For **5**, the HMBC correlations (Table 2) between H-6 and C-11a, H-7β and C-6a, H-14a and C-13a, H-15 and C-3b, H-15 and C-13a (four-bond enhancement), H-16 and C-6a, and H-16 and C-10a (four-bond enhancement) confirmed the assignments of C-11a, C-6a, C-13a, C-3b, and C-10a. Compound **5** was a dimer of the derivative of **3**, however, the assignments of C-3 and C-7, and C-3b and C-6a were interchangeable because of the overlapping of the chemical shifts among these carbons. In **6**, the HMBC correlations (Table 2) between H-6 and C-11a, H-10α and H-10β and C-10b, H-13 and C-4a, H-13 and C-13a (two-bond enhancement), H-14a and C-13a, H-16 and C-10b (four-bond enhancement) confirmed the assignments of C-11a, C-10b, C-4a, and C-13a. Unfortunately, very few correlations for the central, heteroaromatic portion of the molecules were observed in the HMBC spectra. An NH HMBC experiment[15] (J_{NH} = 2 Hz) failed to provide any useful information to confirm the assignments of the quaternary carbons in the central portion of these molecules.

As mentioned earlier, there were two possibilities for the arrangement of two molecules of the derivative of **3** to form isomers **5** and **6**. The overall shapes of the CD spectra of the two isomers were different, and when compared with that of **3**,[14] the CD data of isomer **6** were similar to those of **3**. Therefore, it is tentatively proposed that isomer **6** was formed from two molecules of the derivative of **3** that were in the same orientation and that these two molecules retained the CD properties of **3**. In contrast, two opposite orientations of the derivatives of **3** formed the isomer **5**, which demonstrated a quite different CD spectrum from that of **3**.

The proposed structures of these two blue compounds are different from that postulated for eserine blue (**4**) by Auterhoff and Hamacher[6] in 1967 due to the absence of the carbonyl and one of the *N*-methyl groups and the presence of one extra aromatic proton in these two compounds. However, the unambiguous differentiation of the structures of isomers **5** and **6** by NMR and CD data was not possible. Further studies with model compounds to distinguish between the structures of **5** and **6**, and the mechanism of formation are presently under investigation.

Compounds **5** and **6** were evaluated for their cytotoxic activity using a battery of human cancer cell lines and were judged to be inactive.

Experimental Section

General Experimental Procedures. Physostigmine was purchased from Sigma Chemical Co. Column chromatography utilized Al_2O_3 (neutral, Brockman Activity 1) (80−200 mesh, Fisher), and TLC, Al_2O_3 (neutral) (Merck). TLCs were viewed under a UV lamp (Chromato-Vue C-70 G UV Viewing System). HPLC (Water LC Module I system with a 996 Photodiode Array Detector (set at 610 nm) add-on, model 600 pump, autoinjector), RP-18 250 × 10 mm column, eluted with 0.1% HOAc in DI H_2O−CH_3CN (5:95). CD spectra were measured on a JASCO J-710 CD/ORD spectropolarimeter. UV spectra were obtained in MeOH, using a Beckman DU-7 spectrophotometer, and IR spectra on a Midac Collegian FT-IR spectrophotometer. 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. The ^{1}H-NMR (499.8 MHz), ^{13}C-NMR (125.7 MHz), COSY, HMQC, HMBC, NH-HMBC, and NOESY experiments were performed with a Varian Unity 500 MHz NMR spectrometer. LRMS and HRMS were obtained using a Finnigan MAT 90 mass spectrometer operating at 70 eV.

Preparation of Rubreserine. Rubreserine (**3**) was prepared from physostigmine (**1**) according to the method of Robinson.[9]

Reaction of Rubreserine (3) with NH_3. Rubreserine (**3**) (95.7 mg) was dissolved in absolute EtOH (8 mL) and was refluxed under NH_3 gas for 4.5 h. The reaction mixture was worked up in $CHCl_3$ and dried under vacuum to afford a residue that was subjected to column chromatography.

Isolation of Compounds 5 and 6. Elution of the residue with $CHCl_3$ from an Al_2O_3 (neutral) column yielded fractions 23−29, which, when combined and further purified on HPLC, gave compound **5** (R_f 0.36; $CHCl_3$−MeOH, 5:0.5) as a blue solid (3.5 mg), and fractions 30−32, which gave compound **6** also as a blue solid (5.3 mg) with the same R_f value ($CHCl_3$−MeOH, 5:0.5).

Compound 5: a blue solid; CD $[\theta]_{219}$ −89 738, $[\theta]_{254}$ −6101, $[\theta]_{281}$ −6899; UV (MeOH) λ_{max} (log ϵ) 610 (4.28) nm; IR (film) ν_{max} 2926, 1625 (C═N), 1494, 1298 cm^{-1}; ^{1}H NMR ($CDCl_3$, 499.8 MHz) δ 7.18 (1H, s, H-6), 6.89 (1H, s, H-4), 6.79 (1H, br s, H-13), 6.16 (1H, s, H-11), 4.90 (1H, s, H-9a), 4.31 (1H, s, H-14a), 3.08 (3H, s, H-18), 2.80 (1H, m, H-8β), 2.75 (2H, t, J = 6.0 Hz, H-2α and H-2β), 2.65 (3H, s, H-17), 2.60 (3H, s, H-19), 2.49 (1H, m, H-8α), 2.03 (1H, t, J = 6.0 Hz, H-3β), 2.01 (1H, s, H-7α), 1.98 (1H, t, J = 6.0 Hz, H-3α), 1.86 (1H, m, H-7β), 1.47 (3H, s, H-15), 1.45 (3H, s, H-16); ^{13}C NMR ($CDCl_3$, 125.7 MHz) δ 166.0 (s, C-13a), 150.7 (s, C-5a), 149.8 (s, C-11a), 147.6 (s, C-4a), 138.1 (s, C-3b), 134.6 (s, C-6a), 131.4 (s, C-12a), 128.8 (s, C-10a), 123.1 (d, C-6), 122.2 (d, C-4), 97.4 (d, C-14a), 97.0 (d, C-13), 96.9 (d, C-9a), 90.8 (d, C-11), 53.2 (t, C-2), 52.2 (t, C-8), 51.8 (s, C-3a), 51.2 (s, C-6b), 40.8 (t, C-7), 40.2 (t, C-3), 39.7 (q, C-19), 37.2 (q, C-17), 33.6 (q, C-18), 26.1 (q, C-15), 25.0 (q, C-16); HRESI+ m/z [M + H]$^+$ 416.2452 ($C_{25}H_{30}N_5O$) requires 416.2450; EIMS (70 eV) m/z [M]$^+$ 417 (100), 416 (17), 415 (20), 373 (19), 359 (22), 153 (17), 109 (10).

Compound 6: a blue solid; CD $[\theta]_{224}$ −2897, $[\theta]_{254}$ −1259, $[\theta]_{281}$ −1488, $[\theta]_{342}$ +246, $[\theta]_{383}$ −145, $[\theta]_{414}$ +133; UV (MeOH) λ_{max} (log ϵ) 610 (3.79) nm; IR (film) ν_{max} 2926, 2859, 1625 (C═N), 1494, 1377, 1298 cm^{-1}; ^{1}H NMR ($CDCl_3$, 499.8 MHz) δ 7.10 (1H, s, H-11), 6.70 (1H, s, H-4), 6.60 (1H, s, H-13), 6.08 (1H, s, H-6), 4.82 (1H, s, H-14a), 4.23 (1H, s, H-7a), 3.02 (3H, s, H-16), 2.75 (1H, m, H-2β), 2.71 (2H, t, J = 6.2 Hz, H-9α and H-9β), 2.64 (3H, s, H-19), 2.57 (3H, s, H-17), 2.48 (1H, m, H-2α), 1.99 (2H, t, J = 6.2 Hz, H-10α and H-10β), 1.96 (1H, t, J = 6.2 Hz, H-3α), 1.81 (1H, m, H-3β), 1.43 (3H, s, H-18), 1.41 (3H, s, H-15); ^{13}C NMR ($CDCl_3$, 125.7 MHz) δ 166.9 (s, C-13a), 155.5 (s, C-6a), 149.9 (s, C-5a), 148.0 (s, C-4a), 146.9 (s, C-11a), 138.4 (s, C-12a), 134.7 (s, C-10b), 128.8 (s, C-3b), 122.8 (d, C-11), 121.3 (d, C-4), 99.6 (d, C-14a), 98.7 (d, C-13), 97.5 (d, C-7a), 91.1 (d, C-6), 53.3 (t, C-9), 52.6 (t, C-2), 51.2 (s, C-3a), 51.7 (s, C-10a), 40.7 (t, C-3), 40.2 (t, C-10), 39.6 (q, C-17), 37.5 (q, C-19), 33.7 (q, C-16), 26.2 (q, C-18), 24.7 (q, C-15); HRESI+ m/z [M + H]$^+$ 416.2456 ($C_{25}H_{30}N_5O$) requires 416.2450; EIMS (70 ev) m/z [M]$^+$ 417 (100), 416 (28), 415 (43), 374 (14), 373 (35), 372 (17), 360 (20), 359 (49).

Evaluation of Cytotoxic Activity. Compounds **5** and **6** were evaluated for their cytotoxic activity, using procedures described previously.[16]

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References and Notes

(1) Jobst, J.; Hesse, O. *Ann. Chem.* **1864**, *129*, 115−121.
(2) Hesse, O. *Ann. Chem.* **1867**, *141*, 82−86.
(3) Salway, A. H. *J. Chem. Soc.* **1912**, *101*, 978−989.
(4) Massart, L.; Vandendriessche, L.; Dufait, R. *Enzymologia* **1939**, *7*, 339−341.
(5) Ellis, S.; Krayer, O.; Plachte, F. L. *J. Pharmacol. Exp. Ther.* **1943**, *79*, 309−319.
(6) Auterhoff, H.; Hamacher, H. *Arch. Pharm. Ber. Dtsch. Pharm. Ges.* **1967**, *300*, 849−856.
(7) Ellis, S.; Plachte, F. L.; Straus, O. H. *J. Pharmacol. Exp. Ther.* **1943**, *79*, 295−308.
(8) Ellis, S. *J. Pharmacol. Exp. Ther.* **1943**, *79*, 364−372.
(9) Robinson, B. *J. Pharm. Pharmacol.* **1965**, *17*, 89−91.
(10) Robinson, B. In *The Alkaloids: Chemistry and Physiology*; Manske, R. H. F., Ed.; Academic Press: New York, 1968; Vol. X, pp 383−401.
(11) Schönenberger, B.; Jacobson, A. E.; Brossi, A.; Streaty, R.; Klee, W. A.; Flippen-Anderson, J. L.; Gilardi, R. *J. Med. Chem.* **1986**, *29*, 2268−2273.
(12) Takano, S.; Ogasawara, K. In *The Alkaloids: Chemistry and Pharmacology*; Brossi, A., Ed.; Academic Press: San Diego, 1989; Vol. 36, pp 225−251.
(13) Brossi, A.; Pei, X.-F.; Greig, N. H. *Aust. J. Chem.* **1996**, *49*, 171−181.
(14) Poobrasert, O.; Chai, H.; Pezzuto, J. M.; Cordell, G. A. *J. Nat. Prod.* **1996**, *59*, 1087−1089.
(15) Martin, G. E.; Crouch, R. C.; Sharaf, M. H. M.; Schiff, P. L. *J. Nat. Prod.* **1996**, *59*, 2−4.
(16) You, M.; Wickramaratne, D. B. M.; Silva, G. L.; Chai, H.; Chagwedera, T. E.; Farnsworth, N. R.; Cordell, G. A.; Kinghorn, A. D.; Pezzuto, J. M. *J. Nat. Prod.* **1995**, *58*, 598−604.

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