Synthesis and Preliminary Biological Evaluation of 5-Fluoro-5,8-dideazaisoaminopterin

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The new folate antagonist, 5-fluoro-5,8-dideazaisoaminopterin was synthesized in four steps beginning with 2,4-diamino-5-fluoroquinazoline. It was found to be a potent inhibitor of human dihydrofolate reductase. Against L1210 leukemia in mice, 5-fluoro-5,8-dideazaisoaminopterin was equiactive with methotrexate at approximately one half of the total dose employed.

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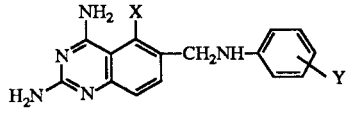
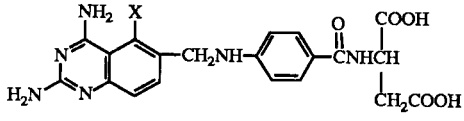
For 2,4-diaminoquinazoline analogues of folic acid significant enhancement of biological activity can often be achieved by the introduction of a small nonpolar group such as methyl or chloro at position five. For example, the antimalarial activity of 2,4-diamino-5-chloro-6-[(3,4-dichloroanilino)methyl]quinazoline, **1b** was 12-fold greater than that of its counterpart devoid of a chlorine at position 5, **1a** [1]. On the other hand, trimetrexate, **1c**, which



1a X = H Y = 3,4-Cl_2
b X = Cl Y = 3,4-Cl_2
c X = CH_3 Y = 3,4,5-$(OCH_3)_3$

contains a methyl group at position 5, was selected from a large group of structurally related compounds, for introduction into clinical trials as an antitumor agent [2-5]. Compound **1c** in combination with calcium leucovorin is also undergoing clinical trials for the treatment of *Pneumocystis carinii* infections [6-8]. Earlier studies with classical 2,4-diaminoquinazoline analogues of folic acid containing a terminal L-aspartate moiety demonstrated that methasquin, **2c**, had more favorable overall pharmacologic properties than chlorasquin, **2b**, or quinaspar, **2a**, [9,10]. Finally, DeGraw and coworkers found that



2a X = H
b X = Cl
c X = CH_3

2,4-diamino-5-methylquinazolines bearing a variety of different alkyl substituents at position six produced synergistic effects against *Mycobacterium* sp. *606* when employed in combination with dapsone [11].

It was of interest, therefore, to examine the effect of other substituents located at position five of the quinazoline ring upon biological activity. This paper describes the synthesis and preliminary antitumor evaluation of 5-fluoro-5,8-dideazaisoaminopterin, **3**.

The synthetic route employed for preparing compound **3** and related compounds is shown in Scheme I. This approach was facilitated by the availability of 2,4-diamino-5-fluoroquinazoline, **4**, in large quantity, which was prepared by the method recently developed in this laboratory [12]. The nitration of **4** gave a mixture of the

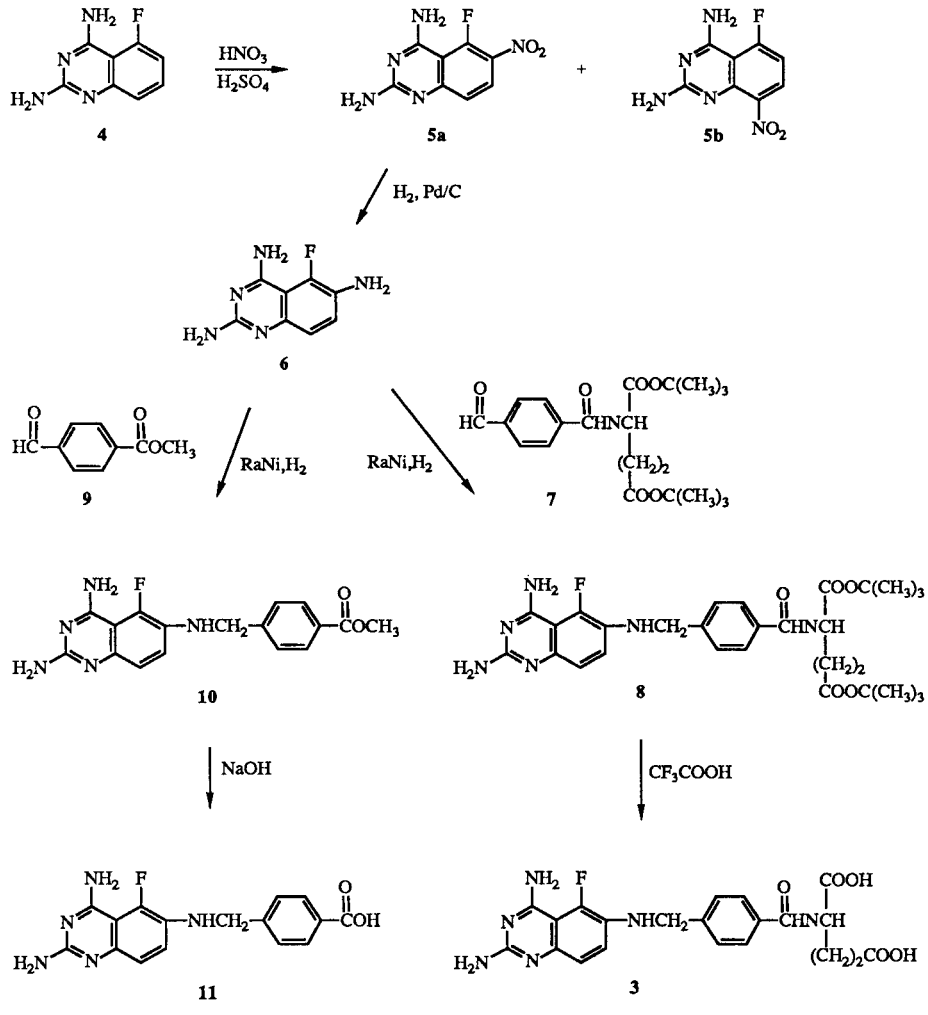
Table 1
Effects of 5-Fluoro-5,8-dideazaisoaminopterin Against L1210 Leukemia in BDF_1 Mice

Compound **3** mg/kg	MTX mg/kg	Treatment (days)	MST (days)	ILS (%)
control	–	–	7.4 (7.5) [a]	–
1.0	–	1, 5, 9	9.0	20
2.0	–	1, 5, 9	11.8	57
4.0	–	1, 5, 9	12.1	61
8.0	–	1, 5, 9	13.8 (14.0) [a]	86 (87) [a]
12.0	–	1, 5, 9	14.6	97
16.0	–	1, 5, 9	15.1	104
–	24.0	1, 5, 9	14.3	93

MST, mean survival time; ILS, increase in mean survival time. Compounds were administered intraperitoneally on the days indicated following intrapeitoneal implantation of 10^6 L1210 cells on day 0. MTX and **3** were dissolved in 0.1 N sodium hydroxide and diluted with 0.9% sodium chloride to achieve the appropriate concentrations for injection.

[a] Numbers in parentheses are values obtained in a replicate experiment.

Scheme I. Synthetic Route to 5-Fluoro-5,8-dideazaisoaminopterin and Related Compounds



6-and 8-nitro isomers (2:1 ratio), **5a** and **5b**, which were resolved chromatographically and their structures proven by nmr spectroscopy. Catalytic hydrogenation of **5a** then afforded the key intermediate, 5-fluoro-2,4,6-triaminoquinazoline, **6**, in satisfactory yield. Compound **6** was then condensed reductively with di-*t*-butyl 4-formylbenzoyl-L-glutamate [13] in the presence of Raney nickel to yield the penultimate di-*t*-butyl ester, **8**. Removal of the *t*-butyl groups with trifluoroacetic acid then gave 5-fluoro-5,8-dideazaisoaminopterin, **3**. Compound **6** was also condensed with methyl 4-formylbenzoate and the resulting product treated with base to give 4-deoxy-4-amino-5-fluoro-5,8-dideazapteroic acid, **11**.

Compound **3** was evaluated against human (WIL2) dihydrofolate reductase (DHFR) and found to be a tight binding inhibitor having an I_{50} of 0.0031 μM. The value obtained for methotrexate (MTX) under these conditions was 0.0043 μM. Against L1210 leukemia cells in culture, **3** exhibited an ED_{50} of 0.03 μM and was, therefore, 6-fold less inhibitory than MTX (ED_{50} = 0.005 μM). The new folate antagonist was also evaluated against L1210 leukemia in mice. Preliminary studies using a dose of 10 mg/kg on a Q2D x 5 regimen revealed that **3** was less effective and more toxic than MTX (ILS = 35% versus 124% for MTX). Therefore, a less frequent schedule was employed and the results are presented in Table 1. It will be seen that **3** is equiactive with MTX at approximately one half of the total dose. It should be noted that the dose of MTX used in these experiments was the same as that reported to be optimum for the Q4D x 3 regimen (T/C = 223%; ILS = 123%) using a 10^6 cell inoculum of L1210 cells in D_2BC mice [10]. Since **3** is more effective using the less frequent regimen of administration, it is suggested that this compound is highly retained by cells and that more frequent administration results in greater cellular accumulation and hence latent toxicity.

EXPERIMENTAL

Melting points were determined on a Mel-temp apparatus and are uncorrected. Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN and Atlantic Microlab, Inc., Norcross, GA. Solvation due to water was confirmed by the presence of a broad peak at ca δ 3.5 in the ^{1}H nmr spectra which was transformed into a sharp singlet by addition of deuterium oxide. Silica gel plates (Baker 1B2 - F) were used for tlc determinations. Free acids were assayed on Eastman 13254 cellulose plates. Column chromatographic separations were performed on Kieselgel 60 (70 - 230 mesh) obtained from E. Merck and Co. The uv spectra were determined with a Cary 219 spectrometer in 0.1 *N* phosphate buffer *p*H 7.00. ^{1}H and ^{19}F nmr spectra were recorded on 90 MHz (Varian EM390), 300 MHz (Bruker AM-300) and 400 MHz (Varian VXR-400) instruments. The ^{1}H chemical shifts are presented in ppm with tetramethylsilane as the internal standard. The ^{19}F chemical shifts are presented in ppm with trifluoroacetic acid [a] or fluorotrichloromethane [b] as the internal standard. Fast atom bombardment mass spectra (FAB) were obtained on a Finnigan MAT 212 spectrometer using argon bombardment by Dr. Michael Walla, Chemistry Department, University of South Carolina. The electron impact mass spectra were obtained off probe using a Finnigan 4521 mass spectrometer.

L1210 leukemia cells sensitive to MTX were grown in suspension culture as described previously [14]. Homogeneous DHFR was obtained from human WIL2 cells as described earlier [15]. It was assayed spectrophotometrically at 340 nm by using 9 μM dihydrofolate, 30 μM NADPH, and 0.15 *M* potassium chloride in 0.05 *M* Tris buffer (*p*H 7.4); [DHFR] = 0.0086 μM by MTX titration. The final volume was 1 ml, and the assay was performed at 22° after a preincubation period of 2 minutes. MTX was a gift from Dr. Suresh Kerwar, Laderle Laboratories, Pearl River, NY.

2,4-Diamino-5-fluoro-6-nitroquinazoline (**5a**) and 2,4-Diamino-5-fluoro-8-nitroquinazoline (**5b**).

A mixture of 90% nitric acid (35 ml) and 98% sulfuric acid (35 ml) was stirred and cooled (ice bath) under nitrogen. To this solution was added portionwise 2,4-diamino-5-fluoroquinazoline (5 g, 0.028 mole) during a 20 minute period. After stirring the reaction mixture at 0° for 15 minutes, the ice bath was removed and the light yellow solution was kept at ambient temperature for 4 hours. After this, the reaction mixture was poured onto crushed ice (150 ml). The light yellow suspension was stirred at 0° while being basified with concentrated ammonium hydroxide to *p*H 8.5. After cooling of the suspension in an ice bath for 3 hours, the precipitate was collected by filtration, washed with water and dried *in vacuo* to yield 4.36 g of an orange crystalline solid, which was a mixture of 6-nitro and 8-nitro isomers. The isomers were separated by chromatography on silica gel with ethyl acetate and tetrahydrofuran as eluents, proceeding gradiently from 100% ethylacetate to 50% of tetrahydrofuran in ethyl acetate in 10% increments. The separation gave 2.81 g of 6-nitro and 1.33 g of 8-nitro isomer which were pure by tlc (ethyl acetate:tetrahydrofuran, 8:2). The 6-nitro isomer **5a** was recrystallized from *N*,*N*-dimethylformamide to give 2.49 g (40%) of an orange crystalline solid mp 282-283°; ^{1}H nmr (DMSO-d_6): 300 MHz δ 6.92 (br s, 2H, 2-NH_2), 7.10 (d, 1H, 8-H, J = 9.38 Hz), 7.40 (br s, 1H, 4-NH), 7.90 (br s, 1H, 4-NH), 8.12 (dd, 1H, 7-H); ^{19}F nmr [a] (DMSO): 300 MHz δ -36.1 (d, 1F, 5-F, J = 6 Hz); ms: (m/e) 223 (M^+).

Anal. Calcd. for $C_8H_6FN_5O_2 \cdot 0.08H_2O$: C, 42.78; H, 2.76; N, 31.18. Found: C, 43.12; H, 2.74; N, 30.86.

The 8-nitro isomer **5b** was recrystallized from tetrahydrofuran:methylene chloride, 2:1 to afford 1.01 g (16%) of a yellow crystalline solid mp 235-238°; ^{1}H nmr (DMSO-d_6): 300 MHz δ 6.73 (br s, 2H, 2-NH_2), 6.85 (dd, 1H, 6-H, J = 8.72 Hz, J = 11.19 Hz), 7.15 (br s, 1H, 4-NH), 7.80 (br s, 1H, 4-NH), 8.01 (dd, 1H, 7-H, J = 8.72 Hz, J = 5.45 Hz); ^{19}F nmr [b] (DMSO): 400 MHz δ -102.74 (m, 1F, 5-F); ms: (m/e) 223 (M^+).

Anal. Calcd. for $C_8H_6FN_5O_2$: C, 43.05; H, 2.71; N, 31.38. Found: C, 43.13; H, 2.61; N, 31.00.

5-Fluoro-2,4,6-triaminoquinazoline (**6**).

A mixture of **5a** (0.46 g, 2 mmoles) and 10% palladium-charcoal (0.160 g) in 2-methoxyethanol (55 ml) was hydrogenated in a low pressure Parr hydrogenator at ambient temperature for 3 hours. After diluting with 2-methoxyethanol (55 ml), the reaction was heated at reflux for 20 minutes, filtered through Celite and the solvent removed under reduced pressure. Trituration of the residual solid with ethyl ether:methylene chloride, 1:1 gave 0.353 g of brown solid. This was purified by chromatography on silica gel with gradient elution, proceeding from 100% ethyl acetate to ethyl acetate:*N*,*N*-dimethylformamide, 7:3 with 10% increments. Evaporation of fractions homogenous by tlc (ethyl acetate:*N*,*N*-dimethylformamide, 7:3) gave 0.320 g of a brown solid which was recrystallized from *N*,*N*-dimethylformamide:-ethyl acetate, 1:10 to yield 0.282 g (71%) of a brown crystalline powder mp 227-229°; ^{1}H nmr (DMSO-d_6): 300 MHz δ 4.82 (br s, 2H, 6-NH_2), 5.67 (br s, 2H, 2-NH_2), 6.91 (d, 1H, 8-H, J = 8.87 Hz + br s, 2H, 4-NH_2), 7.15 (dd, 1H, 7-H); ^{19}F nmr [b] (DMSO): 90 MHz δ 137.2 (br s, 1F, 5-F); ms: (m/e) 193 (M^+).

Anal. Calcd. for $C_8H_8FN_5$: C, 49.73; H, 4.17; N, 36.26. Found: C, 49.33; H, 4.15; N, 35.93.

Di-*t*-butyl *N*-(4-(((2,4-Diamino-5-fluoro-6-quinazolinyl)amino)-methyl)benzoyl)-L-glutamate (**8**).

A mixture of **6** (1.58 g, 8 mmoles), di-*t*-butyl *N*-(4-formylbenzoyl)-L-glutamate [13] (4.65 g, 12 mmoles) and Raney nickel (*ca* 1.5 g) in 70% acetic acid (100 ml) was hydrogenated in low pressure Parr hydrogenator at ambient temperature for 15 hours. The reaction mixture was treated with charcoal, stirred at room temperature for 30 minutes and filtered through Celite. The filtrate was cooled and basified with concentrated ammonium hydroxide to *p*H 8.5. After cooling the suspension at 0° for 4 hours, the precipitate was collected by filtration, washed with water and dried *in vacuo*. The residual solid was purified on a silica gel column, which was eluted by chloroform:methanol proceeding from pure chloroform to chloroform:methanol, 9:1 with 2% increments of methanol. Fractions, homogeneous by tlc (chloroform:methanol, 8:2) were evaporated under reduced pressure to yield 2.71 g of a green solid. This material was recrystallized from carbon tetrachloride:*n*-hexane, 1:1, to give 2.11 g (45%) of a light green powder mp 105-107°; ^{1}H nmr (DMSO-d_6): 300 MHz δ 1.37 (s, 9H, $C(CH_3)_3$), 1.39 (s, 9H, $C(CH_3)_3$), 1.83-2.10 (m, 2H, β-CH_2), 2.32 (t, 2H, γ-CH_2), 4.31 (m, 1H, α-CH), 4.44 (br s, 2H, $NHCH_2$), 6.15 (br t, 1H, $NHCH_2$), 6.31 (br s, 2H, 2-NH_2), 6.90 (d, 1H, 8-H, J = 9.08 Hz), 7.02 (dd, 1H, 7-H), 7.45 (d, 2H, 3′-H, 5′-H, J = 8.28 Hz), 7.50 (br s, 2H, 4-NH_2), 7.80 (d, 2H, 2′-H, 6′-H, J = 8.28 Hz), 8.51 (d, 1H, CONH, J = 7.45 Hz); ^{19}F nmr [b] (DMSO): 400 MHz δ -134.31 (br s, 1F, 5-F); ms: (FAB) (m/e) 569 $(M+1)^+$.

Anal. Calcd. for $C_{29}H_{37}FN_6O_5 \cdot 0.25H_2O$: C, 60.77; H, 6.60; N, 14.66. Found: C, 60.76; H, 6.33; N, 14.72.

N-(4-(((2,4-Diamino-5-fluoro-6-quinazolinyl)amino)methyl)benzoyl)-L-glutamic Acid (**3**).

A solution of **8** (2.71 g, 5 mmoles) in 99% trifluoroacetic acid (65 ml) was stirred at room temperature for 1.5 hours under nitrogen. The trifluoroacetic acid was evaporated under reduced pressure and the residual semisolid triturated with ethyl ether:methylene chloride, 1:1 to give a precipitate which was collected by centrifugation. The pellet was resuspended in water (50 ml) and basified with 1*N* sodium hydroxide to *p*H 11.8. Insoluble impurities were removed by filtration and the filtrate brought to *p*H 3.4, first with concentrated and then with 1*N* hydrochloric acid. The precipitate was collected by centrifugation, washed sequentially with acetone (3 x 80 ml), ethyl ether (3 x 80 ml), *n*-pentane (80 ml) and dried *in vacuo* to yield 1.86 g (85%), of a green crystalline solid, pure by tlc (cellulose, 5% ammonium bicarbonate) mp 218-220°; uv: λ max 246 nm (ε 53, 595), λ max 364 nm (ε 4, 347), λ min 216 nm (ε 24, 266), λ min 320 (ε 2, 670); ^{1}H nmr (DMSO-d_6): 300 MHz δ 1.85-2.15 (m, 2H, β-CH_2), 2.29 (t, 2H, γ-CH_2), 4.32 (m, 1H, α-CH), 4.45 (br s, 2H, $NHCH_2$), 6.30 (br s, 1H, $NHCH_2$), 6.96 (d, 1H, 8-H, J = 8.00 Hz), 7.05 (dd, 1H, 7-H, J = 8.00 Hz), 7.30 (br s, 2H, 2-NH_2), 7.44 (d, 2H, 3′-H, 5′-H, J = 8.07 Hz), 7.81 (d, 2H, 2′-H, 6′-H, J = 8.07 Hz), 7.91 (br s, 2H, 4-NH_2), 8.28 (d, 1H, CONH, J = 8.00 Hz); ^{19}F nmr [a] (DMSO): 300 MHz δ -56.94 (br s, 1F, 5-F); ms: (FAB) (m/e) 455 (M-1).

Anal. Calcd. for $C_{21}H_{21}FN_6O_5 \cdot H_2O$: C, 53.16; H, 4.89; N, 17.72. Found: C, 53.53; H, 4.65; N, 17.70.

Methyl 4-(((2,4-Diamino-5-fluoro-6-quinazolinyl)amino)methyl)benzoate (**10**).

A mixture of **6** (0.210 g, 1.1 mmoles) and methyl *p*-formylbenzoate (0.213 g, 1.29 mmoles) in 70% acetic acid (25 ml) was hydrogenated at low pressure in the presence of Raney nickel (*ca* 2.17 g) until hydrogen uptake ceased. The process was repeated with additional methyl *p*-formylbenzoate (0.213 g, 1.29 mmoles) and Raney nickel (*ca* 2.17 g). The resulting reaction mixture was treated with charcoal and stirred at room temperature for 30 minutes. Filtration through Celite gave a clear solution, which was basified with concentrated ammonium hydroxide to *p*H 8. A green solid was collected by filtration, washed with water, *n*-hexane, and *n*-pentane, and then dried *in vacuo*. Chromatography on silica gel with chloroform:methanol, 9:1 as the eluent gave a green solid, pure by tlc (chloroform:methanol, 8:2). Recrystallization from chloroform:*n*-hexane, 9:1 afforded 0.255 g (69%) of a green crystalline solid, mp 205-206°; ^{1}H nmr (DMSO-d_6): 400 MHz δ 3.83 (s, 3H, CH_3), 4.45 (d, 2H, $NHCH_2$, J = 4.82 Hz), 5.75 (br s, 2H, 2-NH_2), 5.90 (br t, 1H, $NHCH_2$, J = 4.82 Hz), 6.84 (d, 1H, 8-H, J = 8.97 Hz), 6.90 (br s, 2H, 4-NH_2), 6.97 (dd, 1H, 7-H), 7.50 (d, 2H, 3′-H, 5′-H, J = 8.22 Hz), 7.90 (d, 2H, 2′-H, 6′-H, J = 8.22 Hz); ^{19}F nmr [a] (DMSO): 300 MHz δ -24.48 (d, 1F, 5-F, J = 8.24 Hz); ms: (FAB) (m/e) 342 $(M+1)^+$.

Anal. Calcd. for $C_{17}H_{16}FN_5O_2$: C, 59.82; H, 4.73; N, 20.52. Found: C, 59.53; H, 4.78; N, 20.40.

4-(((2,4-Diamino-5-fluoro-6-quinazolinyl)amino)methyl)benzoic Acid (**11**).

A suspension of **10** (0.3 g, 0.8 mmoles) in 0.2*N* sodium hydroxide (30 ml) was stirred at ambient temperature for 67 hours. The reaction mixture was filtered on a sintered glass funnel and filtrate acidified with concentrated hydrochloric acid to *p*H 6 to give a light brown precipitate. Centrifugation gave a pellet which was washed with water (2 x 20 ml) and acetone (20 ml) by cycles of resuspension, centrifugation, and decantation. After drying *in vacuo*, the solid was resuspended in chloroform (50 ml), stirred at ambient temperature for 1 hour, then filtered off, rinsed sequentially with ethyl ether (2 x 30 ml), *n*-pentane (2 x 30 ml) and dried *in vacuo* to yield 0.244 g (85%) of light brown crystalline product mp >355°; tlc (chloroform:methanol, 8:2); ^{1}H nmr (DMSO-d_6): 300 MHz δ 4.42 (br s, 2H, $NHCH_2$), 6.08 (br s, 1H, $NHCH_2$), 6.50 (br, s, 2H, 2-NH_2), 6.92 (d, 1H, 8-H, J = 8.32 Hz), 7.04 (dd, 1H, 7-H, J = 8.32 Hz), 7.07 (br s, 2H, 4-NH_2), 7.42 (d, 2H, 3′-H, 5′-H, J = 7.99 Hz), 7.85 (d, 2H, 2′-H, 6′-H, J = 7.99 Hz); ^{19}F nmr [a] (DMSO): 300 MHz δ - 24.72 (d, 1F, 5-F, J = 8.42 Hz); ms: (FAB) (m/e) 328 $(M+1)^+$.

Anal. Calcd. for $C_{16}H_{14}FN_5O_2 \cdot H_2O$: C, 55.65; H, 4.67; N, 20.28. Found: C, 55.39; H, 4.67; N, 20.32.

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