STRUCTURE AND SYNTHESES OF TEXAZONE, 2-(*N*-METHYLAMINO)-3*H*-PHENOXAZIN-3-ONE-8-CARBOXYLIC ACID, AN ACTINOMYCETE METABOLITE

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(Received for publication January 22, 1983)

Cultures of actinomycete strain WRAT-210 produced a dark red crystalline metabolite which was named texazone. Spectroscopic evidence suggested that the structure of texazone is 2-(*N*-methylamino)-3*H*-phenoxazin-3-one-8-carboxylic acid. The structure was confirmed by chemical synthesis through oxidative dimerization of ethyl 3-amino-4-hydroxybenzoate with 2-(*N*-methylamino)phenol and subsequent hydrolysis of the resultant phenoxazinone ester.

Derivatives of 2-amino-3*H*-phenoxazin-3-one (**1**) first found in arthropods and molluscs (ommochromes[1]) have also been isolated from fungi[2~4] and actinomycetes[5]. We report here the isolation of a new pigment (texazone) of this type from an actinomycete. The structure **3** was suggested by spectroscopic methods and confirmed by chemical syntheses.

Fig. 1. 2-Amino-3*H*-phenoxazin-3-one.

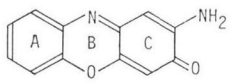

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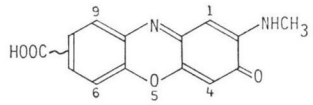
Fig. 2. Possible structures for texazone.



2 HOOC at C-7
3 HOOC at C-8 (Texazone)

Production and Characterization

The actinomycete which produces texazone was obtained from a Texas soil as isolate WRAT-210 and is currently under taxonomic study. Texazone was produced when the culture was grown in a glucose - soy bean meal - medium and was extracted from acidified broth with ethyl acetate. After purification by column chromatography on silica gel and crystallization from acetone, texazone was obtained as red needles which decomposed without melting at 240°C; λ_{max} in methanol at 247, 257, 426, 436 nm and a broad inflexion at 480~490 nm (log ε: 4.34, 4.37, 4.20, 4.21 and 3.70); ν_{max} in KBr at 3395, 3335, 3050, 2920, 2815, 1678, 1625, 1585, 1495, 1425, 1335, 1300, 1266, 1200, 1182, 1115, 1030, 845, 820, 758, and

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702 cm^{-1}. The compound showed no optical activity. It was soluble in trifluoroacetic acid, dimethyl sulfoxide and pyridine, less soluble in ethyl acetate, chloroform, ethanol and ether and insoluble in water.

The mass spectrum of texazone showed a molecular ion at *m/z* 270 which was also the base peak. Significant fragment ions were observed at *m/z*, 254 (25%), 243 (13), 242 (22), 225 (4) and 213 (13). Doubly charged ions were found at and below *m/z* 135, the most intense being at *m/z* 112 (12%). The mass of the molecular ion, accurately measured was 270.0637 (calcd. for $C_{14}H_{10}N_2O_4$, 270.0641).

The ^1H NMR of texazone (22 mg) in dimethyl sulfoxide-d_6 (0.4 ml) with tetramethylsilane as an internal standard gave signals at δ (ppm): 2.86 (d, J=4.5 Hz, 3H), 6.10 (s, 1H), 6.20 (s, 1H), 7.29 (m, J=4.5 Hz, 1H), 7.53 (d, J=8.5 Hz, 1H), 7.96 (dd, J=1.8 and 8.5 Hz, 1H), and 8.14 (d, J=1.8 Hz, 1H). In trifluoroacetic acid-*d*, signals of similar relative intensities occurred at δ 3.42, 6.60, 6.87, 7.72, 8.37 and 8.44; similar couplings were observed except for the signal at δ 3.42, which was now a singlet.

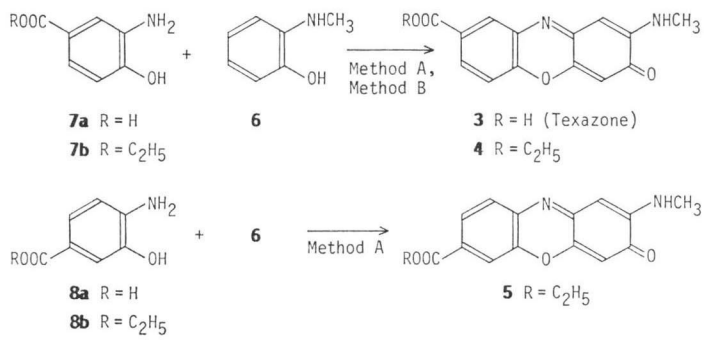
The electronic absorption spectrum of texazone closely resembled that of 2-amino-3*H*-phenoxazin-3-one (**1**). For both compounds, acid shifts the visible maxima hypsochromically and enhances the broad envelope centered at 500 nm. Texazone differs from **1** in possessing an acidic function, which is apparent from its solubility in 0.1 N sodium hydroxide and from the absorption maxima at 1678 cm^{-1} due to carbonyl stretching and in the range from 2815 to 3395 cm^{-1} associated with the hydrogen-bonded hydroxyl group of a carboxyl function. The ion at *m/z* 225 also suggests loss of a carboxyl group from the metabolite during electron-impact mass spectrometry.

Considering the molecular formula of texazone, $C_{14}H_{10}N_2O_4$, a parent 2-amino-3*H*-phenoxazin-3-one structure, **1**, would account for $C_{12}H_8N_2O_2$. One of the two remaining carbon atoms is associated with the carboxyl function, thus there is a need to account for only an additional carbon and 2 hydrogen atoms. The two doublet signals at δ 2.86 (3H) and 7.29 (1H) in the NMR spectrum indicated that there was a methyl group attached to the amino substituent at C-2[6]. In dimethyl sulfoxide-d_6, these are coupled (J=4.5 Hz). As would be expected for a CH_3NH-substituent, the spectrum in trifluoroacetic acid-*d* showed only a singlet at δ 3.42 for the methyl group; the NH-proton had rapidly equilibrated with deuterium. Similar results were obtained by adding D_2O or CD_3OD to a dimethyl sulfoxide-d_6 solution of texazone. Two one-proton singlets at δ 6.10 and 6.20 are assigned to H-1 and H-4, respectively, by analogy with the assignments for hydrogens at these positions in **1**. Signals at δ 7.53, 7.96 and 8.14 are assigned to H-6, H-7 and H-9 respectively, for the following reasons: the hydrogens in ring-A of **1** are approximately 1.5 ppm downfield of those in ring C; the carboxyl group in texazone, which must be located in ring A, would cause a further downfield shift of the two adjacent hydrogens. The coupling constant of 8.5 Hz between the doublets at δ 7.53 and 7.96 suggests that the two hydrogens are adjacent and separated from the hydrogen responsible for δ 8.14 doublet which is coupled less strongly (J=1.8 Hz) to the δ 7.96 hydrogen. Therefore, the carboxyl group must be at C-7 (**2**) or C-8 (**3**) as shown in Fig. 2. The C-8 position is favored because it allows the lowest-field ring A hydrogen to be located at C-9, *peri* to the nitrogen at position 10. Assignment of the δ 7.53 signal to H-6 is consistent with known substituent effects.[7].

Synthesis

Several one electron transfer oxidants, including air, have been used for the oxidative dimerization of 2-aminophenols to 2-amino-3*H*-phenoxazin-3-ones[8]. In our hands the feasibility of a given phenoxazinone synthesis could be ascertained by superimposing spots of reactant 2-aminophenols on silica TLC plates. After several days exposure to air, but protected from light, followed by development of the

Fig. 3. Outline of syntheses.



Method A : PbO_2/C_2H_5OH, Method B : air/TLC silica plate

plate in a suitable solvent system, if spots of the desired 2-amino-3*H*-phenoxazin-3-one(s) were seen then a successful larger scale synthesis could be expected. For this diagnostic procedure MN Polygram SIL G/UV 254 plates were used.

A study of the literature revealed multi-step syntheses of 2-(*N*-methylamino)phenol (**6**) and suggested that it might not be stable to storage[9]. Therefore exploratory oxidative dimerizations were carried out with 2-aminophenol instead of **6**. The final procedure for the synthesis of **6** was modeled after a briefly described method in which **6** was prepared in one step and used without isolation[10].

In preliminary experiments using silica TLC plates in air[11], amino acids **7a** and **8a** failed to condense with 2-aminophenol, presumably due to their zwitterionic character. Attempts to prepare the methyl esters of **7a** and **8a** in methanol - sulfuric acid were unsuccessful because of the poor solubility of their sulfuric acid salts in methanol. However, the ethyl esters (**7b** and **8b**) were readily made by such a procedure. Both condensed with 2-(*N*-methylamino)phenol (**6**) in ethanol using lead dioxide[10] (method A). Using silica TLC plates in air[11] (method B) ester **7b** gave milligram amounts of the texazone ester (**4**) but **8b** did not react with **6**. Trace amounts of texazone (**3**) were obtained directly from the acid **7a** and **6** using lead dioxide.

TLC of the reaction mixtures revealed that the rate of formation of the desired phenoxazinones **3**, **4** and **5**, was slow. Products from competing reactions could be seen, especially 2-amino-3*H*-phenoxazin-3-one from the dimerization of 2-aminophenol and 2-(*N*-methylamino)-3*H*-phenoxazin-3-one from the condensation of 2-aminophenol with **6**. The mixtures proved difficult to separate: thus, using method A, the yields of **4** (about 2%) and **5** (less than 1%) represent isolated pure material. In both cases additional product was present in mixed fractions. Esters **4** and **5** were readily differentiated on the basis of melting points, IR, NMR, UV-visible spectra and TLC.

The aqueous hydrochloric acid hydrolysis of **4** gave an essentially quantitative yield of **3** after 2.5 hours at 95°C. Texazone was identical with **3** in all spectral properties and by TLC in seven different solvent systems. All attempts to hydrolyze **5** to the 7-carboxylic acid were unsuccessful.

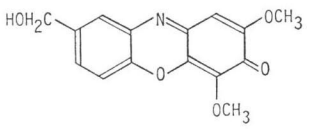
Discussion

Wherever the biosynthesis of 2-amino-3*H*-phenoxazin-3-ones has been investigated they have been shown to originate from oxidative condensation of 2-aminophenols. The parent substance, **1**, and two simple derivatives substituted on the amino nitrogen, have been isolated from cultures of several actinomycetes but more commonly a naturally occurring 3*H*-phenoxazin-3-one will have carbon substi-

tuents at C-1 and C-9[2,3]. Then the biosynthetic precursor is assumed to be 3-hydroxyanthranilic acid or an appropriate derivative formed either directly or indirectly from tryptophan, *via* anthranilic acid. This has been verified experimentally, most notably with the actinomycins, where the immediate precursor is a 3-hydroxy-4-methylanthranoyl peptide and the amino-3*H*-phenoxazin-3-one metabolite bears not only peptidolactone substituents at C-1 and C-9 but also methyl groups at C-4 and C-6[12].

Texazone is the second example of a phenoxazinone metabolite with a substituent at C-8. Michigazone, Fig. 4, isolated from *Streptomyces michiganensis* has a hydroxymethyl group in this position[13]. In both molecules ring A contains a *meta* $C\text{-}C_6\text{-}N$ skeleton, suggesting that the precursor aminophenol may be of different biosynthetic origin from that in the actinomycins and related anthranilate-derived products. A related $C\text{-}C_6\text{-}N$ compound, 3-amino-5-hydroxybenzoic acid is an intermediate in the biosynthesis of mitomycin and the ansamycin antibiotics[14~16] and has recently been shown to occur naturally in *S. verticillatus*[17].

Fig. 4. Michigazone.



Texazone was previously named "actin-red"[18].

Experimental

The electron-impact mass spectrum was obtained with a Dupont/CEC model 21-491 spectrometer. For the accurate mass measurement of the molecular ion a Dupont/CEC model 21-110B was used with perfluorokerosene as reference. For texazone a Varian model HA-100 was used to obtain the ^1H NMR spectra whereas, for the synthetic 3*H*-phenoxazin-3-ones, the instrument was a Varian XL 100-15. Unless otherwise noted MN Polygram SIL G/UV 254 precoated plastic sheets were used for TLC and Hi-flosil Silica gel 60/200 mesh, Applied Science Lab. Inc. State College, PA., for column chromatography.

Production and Isolation of Texazone

Actinomycete WRAT-210 was maintained on agar slants containing (g/liter); glucose 4.2, peptone 5, yeast extract 1.5, beef extract 1.5, NaCl 3.5, K_2HPO_4 3.68, KH_2PO_4 1.32, agar 15. To prepare a vegetative inoculum, a slant culture was transferred to a 250-ml Erlenmeyer flask with 50 ml of a medium consisting of (1 liter); sucrose 30 g, corn steep liquor 20 ml, $CaCO_3$ 7 g, $(NH_4)_2SO_4$ 2 g. After a 5-day incubation at 27°C on a rotary shaker (3.8 cm eccentricity, 220 rpm), 5-ml portions were used to inoculate each 50 ml of production medium. To produce texazone, cultures were grown in a medium consisting of (g/liter) glucose 40, soybean meal 25, and $CaCO_3$ 4. Incubation conditions were the same as used for the vegetative inoculum.

The filtrate from 8-day old cultures was adjusted to pH 3 with hydrochloric acid and extracted with ethyl acetate. Evaporation of solvent from the extract gave a red-brown gum which was chromatographed on a column of silica gel with a stepped gradient of ethyl acetate in benzene as eluant. The fraction collected during elution with benzene - ethyl acetate (3: 1) was concentrated to dryness and the residue recrystallized from acetone.

2-(*N*-Methylamino)-3*H*-phenoxazin-3-one-8-carboxylic Acid (**3**, Texazone)

(a) 2-(*N*-Methylamino)phenol (**6**)[10]: A solution of 0.55 g (5.05 mmole) of freshly recrystallized 2-aminophenol in 8 ml of 95% ethanol was obtained on warming and stirring; cooling resulted in the formation of a fine suspension. To that suspension was added at room temperature, dropwise, 0.65 g (5.28 mmole) of methyl iodide dissolved in 2 ml of 95% ethanol. A clear solution formed only after 10 minutes of additional stirring. The stirring was continued for an additional 24 hours at room temperature. TLC showed the presence of 2 components whose Rf's (0.83 and 0.88) differed from that of 2-aminophenol (Rf 0.77) in C_6H_6 - MeOH - Me_2CO, 14: 3: 3.

(b) Ethyl 2-(*N*-Methylamino)-3*H*-phenoxazin-3-one-8-carboxylate (**4**). Method A, from **7b**: To a stirred solution of freshly recrystallized ethyl 3-amino-4-hydroxybenzoate (**7b**), 0.455 g (2.5 mmole), in 5.0 ml of the solution from (a) [*ca.* 2.5 mmole of 2-(*N*-methylamino)phenol] was added 0.75 g (3.14 m-

mole) of lead dioxide. Stirring was continued at room temperature, in the dark, for 48 hours. At that time TLC using the same solvent as in (a) indicated the presence of at least five closely related components, Rf 0.72~0.91. The mixture was heated to boiling, filtered, and the insoluble material reextracted with two-12.5 ml portions of boiling 2-propanol. The second extract was essentially colorless. The solid which crystallized from the combined filtrates was filtered to give 19.9 mg of dark red solid (A). The filtrate was concentrated to one-half volume, kept at room temperature for 3 days, and again filtered to give 12.9 mg of additional dark red solid (B). The second filtrate when concentrated to dryness gave a red crystalline residue from which 0.113 g of unreacted **7b** (30% recovery) was obtained by extraction with boiling benzene.

Solids A and B, 32.8 mg, were combined and chromatographed on a column of silica gel (200 g) prepared in benzene. Elution with 750 ml of C_6H_6 - Me_2CO, 3: 1 gave 500 ml of colorless forecuts followed by three-100 ml reddish orange fractions. The latter contained a single component as shown by TLC in two solvent systems: Rf 0.76 (C_6H_6 - Me_2CO, 3: 1) and Rf 0.27 (C_6H_6 - $CHCl_3$ - Me_2CO, 9.5: 9.5: 0.5). Those three fractions were combined and concentrated to dryness to give 12.0 mg (1.6% yield) of **4**; mp 256~257°C; λ_{max} (EtOH) 250 (sh), 265, 412, 431 nm; (EtOH - HCl) 247, 258, 433, 518 (sh) nm; (AcOEt) 403, 423 nm; IR ν (mull) 3360 (NH), 1725 (CO) cm^{-1}; (KBr) 3250, 1725 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.42 (t, J=7 Hz, 3H, CH_3CH_2), 2.99 (d, J=6 Hz, 3H, NCH_3), 4.40 (q, J=7 Hz, 2H, CH_3CH_2), 5.76~6.06 (m, 1H, NH), 6.16 (s, 1H, H-1), 6.40 (s, 1H, *H*-4), 7.38 (d, J=8 Hz, 1H, H-6), 8.05 (dd, J=2, 8 Hz, 1H, H-7), 8.41 (d, J=2 Hz, 1H, H-9); MS 298 (M^+ and base peak), 270, 254, 243, 242, 225.

Anal. Calcd. for $C_{16}H_{14}N_2O_4$: C 64.42, H 4.74, N 9.39.
Found: C 64.63, H 5.01, N 9.29.

(c) Method B, from **7b**: To 3 mg of **6** in 1 ml of 95% ethanol, prepared by dilution of the solution of **6** described above, was added 2 mg of **7b** in 1 ml of 95% ethanol. The solution was applied over the entire surface of a 20×10 cm silica gel plate which had been laboratory-made from Silica gel 60 PF-254 (EM Reagents, E. Merck, Darmstadt, Germany), activated one hour at 110°C, then kept exposed to air for several months.

After one week in air but protected from light, the dark brown silica was scraped off and extracted batchwise with ethyl acetate. Analytical TLC using benzene - acetone, 3: 1 disclosed four brown spots, Rf 0.73, 0.66, 0.56 and 0.44. The two faster-moving spots were red-brown, the slower ones yellow-brown. TLC indicated that the spot at Rf 0.44 was **1** by comparison with an authentic sample and that at Rf 0.56 was ethyl 2-amino-3*H*-phenoxazin-3-one-8-carboxylate, since it was identical with the product obtained from a similar reaction between **7b** and 2-aminophenol; the Rf 0.66 spot was 2-(methylamino)-3*H*-phenoxazin-3-one, identical with the product obtained from a similar reaction between **6** and 2-aminophenol; the Rf 0.73 spot was identical with **4** from method A.

The four phenoxazinones were separated by silica gel column chromatography using benzene and 2~5% acetone in benzene as eluant and monitoring eluate fractions by TLC. Crystalline **4** was obtained from combined concentrated, eluate fractions which by TLC analysis contained only **4**. The order of elution from the column was 2-(*N*-methylamino)-3*H*-phenoxazin-3-one, **4**, **1**, and then ethyl 2-amino-3*H*-phenoxazin-3-one-8-carboxylate. This order of migration was also observed on TLC using benzene - chloroform - acetone, 19: 19: 1.

Pure 2-*N*-(methylamino)-3*H*-phenoxazin-3-one had mp 227~229°C (Ref. mp 209~211°C)[10], λ_{max} ($CHCl_3$) 442, 423 (sh) nm; (EtOH) 438, 423, 240 (sh), 238 nm; (EtOH - HCl) 515 (sh), 460 (broad), 270 (sh), 238 nm; IR ν (mull) 3360 (NH), 2920 (CH), 1640, 1620, 1570~1590, 1485, 1420, 1200, 1175, 845, 750 cm^{-1}. Ethyl 2-amino-3H-phenoxazin-3-one-8-carboxylate, not previously reported had mp 220~223°C, λ_{max} (AcOEt) 423, 403; (EtOAc - EtOH - HCl) 455 (broad) nm; IR ν (mull) 3400 & 3300 (NH), 2900 (CH), 1700 (ester CO), 1595~1570, 1260, 1200, 1170, 845, 750 cm^{-1}.

(d) Hydrolysis of **4** to **3**: A solution of 11.0 mg (3.37 mmole) of **4**, 6 ml of concentrated hydrochoric acid (37%) and 24 ml of water in an open-neck flask was heated for 2.5 hours at 95°C (internal temperature) while maintaining the original volume by the addition of water. The solution was then concentrated to dryness, *in vacuo*, on a rotary evaporator, the residue was stirred with 4 ml of water, centrifuged, the supernatant decanted, and the solid dried *in vacuo* to give 10.0 mg (quantitative yield)

of **3**. TLC in each of the following seven solvent systems showed a single spot identical with that shown by texazone: Rf 0.09 (C_6H_6 - EtOAc, 1: 1); Rf 0.49 (C_6H_6 - EtOAc - H_2O, 1: 1/satd, one phase); Rf 0.58 ($CHCl_3$ - EtOAc - AcOH, 9: 9: 1); Rf 0.1 (C_6H_6 - Me_2CO, 3: 1); Rf 0.65 (C_6H_6 - Me_2CO - AcOH, 15: 5: 0.5); Rf 0.64 (C_6H_6 - Me_2CO - AcOH - H_2O, 15: 5: 0.5/satd, one phase); Rf 0.94 ($CHCl_3$ - MeOH-AcOH, 10: 1: 0.5). The UV, IR, NMR and mass spectra of this material were identical with those from texazone.

Texazone (**3**). Method A, from **7a**

A sample of 0.385 g (2.5 mmole) of recrystallized **7a** dissolved in 10 ml of 95% ethanol, at the bp, remained a clear solution when allowed to cool to ambient temperature. To that solution was added 5.0 ml of a 2-(*N*-methylamino)phenol solution similar to that used in Method A, followed by 0.75 g (3.14 mmole) of lead dioxide. The mixture was stirred for seven days in the dark at room temperature. The deep red mixture was heated to the bp, filtered hot, and the insoluble material reextracted successively with three-25 ml portions of boiling 2-propanol. The third extract was essentially colorless. The combined alcoholic extracts were examined by TLC in 4 systems: all revealed at least four products, none of which corresponded to **3**.

The dried lead dioxide residue weighed 1.002 g. It was leached with 4~10 ml portions of 6% aqueous hydrochloric acid at room temperature: the first extract was an intense red, the next two were far less colored, while the last extract was a weak pink. The combined acid extracts were concentrated to dryness *in vacuo*. The dried residue (0.2594 g) was extracted successively with 4~10 ml portions of boiling acetone, and the combined filtrates concentrated to 10 ml. The solid which appeared (*ca.* 0.1 mg of dark red crystals) was removed by filtration. TLC in three systems and the electronic absorption spectrum identified the product as **3**, identical with the sample prepared from **4**.

Ethyl 2-(*N*-Methylamino)-3*H*-phenoxazin-3-one-7-carboxylate (**5**)

To a stirred solution of freshly recrystallized ethyl 4-amino-3-hydroxybenzoate (**8b**), 0.455 g (2.5 mmole) in 5 ml of a solution of **6**, prepared as described above, was added 0.75 g (3.14 mmole) of lead dioxide. Stirring continued at room temperature in the dark for 5 days. The mixture was heated to the bp, filtered, and the insoluble material reextracted successively with three-12,5 ml portions of boiling 2-propanol. The solid which separated from the combined filtrates was filtered to give 21.0 mg of shiny red-purple needles. Column chromatography on 200 g of silica gel as described above gave four-100 ml fractions containing a single component, Rf 0.78 (C_6H_6 - Me_2CO, 3: 1). Concentration *in vacuo* yielded 9.8 mg of **5**, mp 214~215°C, λ_{max} (EtOH) 277, 302, 422, 443 nm; (EtOH - HCl) 280, 300 (sh), 448, 517 (sh) nm; IR ν (KBr) 3300 (NH), 1705 (CO) cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.42 (t, J=7 Hz, 3H, CH_3CH_2), 3.01 (dd, J=5, 2 Hz, 3H, N-CH_3), 4.43 (q, J=7 Hz, 2H, CH_3CH_2), 5.80~6.13 (m, 1H, NH), 6.20 (s, 1H, H-1), 6.42 (d, J=2 Hz, 1H, H-4), 7.39 (m, 1H, H-6), 7.75 (apparent d, J=10 Hz, 1H, H-8 or 9), 8.02 (apparent dd, J=2, 8 Hz, 1H, H-8 or 9); Rf 0.81 (C_6H_6 - Me_2CO, 3: 1), 0.90 ($CHCl_3$ - MeOH, 10: 1), 0.80 (C_6H_6 - Me_2CO - AcOH, 15: 5: 1), and 0.84 (C_6H_6 - EtOAc, 1: 1).

Anal. Calcd. for $C_{16}H_{14}N_2O_4$: C 64.42, H 4.74, N 9.39.
Found: C 64.69, H 4.63, N 8.96.

Acknowledgments

The authors thank MARY P. LECHEVALIER for producing broths containing texazone and Magda Gagliardi for technical assistance. This research was supported in part by the Charles and Johanna Busch Bequest.

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