Growth Inhibitory Properties of Aromatic α-Ketoaldehydes toward Bacteria and Yeast. Comparison of Inhibition and Glyoxalase I Activity

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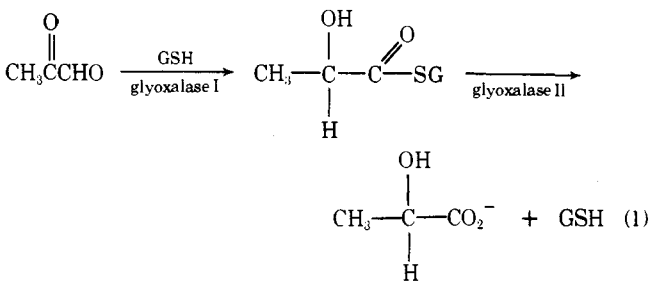
The α-ketoaldehydes methylglyoxal and substituted phenylglyoxals are similar in their abilities to inhibit the growth of *Escherichia coli* and yeast. When logarithmically growing cells are added to media containing 0.3–1 m*M* α-ketoaldehyde, growth stops for several hours, after which normal growth resumes. The period of growth inhibition does not appear to show any correlation with the ability of glyoxalase I to detoxify these α-ketoaldehydes. *E. coli* and yeast glyoxalase I show markedly different substrate specificities. For example, although both enzymes show broad specificity for both aliphatic and aromatic α-ketoaldehydes, 2,4,6-trimethylphenylglyoxal is a substrate for the *E. coli* enzyme but not for the yeast enzyme. Nevertheless, this α-ketoaldehyde inhibits the growth of both *E. coli* and yeast, similar to the other α-ketoaldehydes. Enzymes other than glyoxalase I must play a major role in the metabolism of these α-ketoaldehydes during the period of growth inhibition.

The carcinostatic and general growth inhibitory properties of α-ketoaldehydes are well known, although the physiological functions of naturally occurring α-ketoaldehydes are not understood.[3] Most attention has been given to aliphatic α-ketoaldehydes whose carcinostatic activity was studied extensively by French and Freedlander.[3a] However, as these authors pointed out, the ubiquitous glyoxalase system is able to convert α-ketoaldehydes into nontoxic α-hydroxycarboxylic acids as shown for methylglyoxal in eq 1.[4] Vince and coworkers designed a number of inhibitors of glyoxalase I, some of which appear to be promising anticancer agents.[5] They reasoned that the inhibition of glyoxalase I may allow levels of α-ketoaldehydes to build up, thereby providing an intracellular source of α-ketoaldehydes for growth inhibition. Szent-Gyorgyi and coworkers have proposed that a derivative of an α-ketoaldehyde is required for normal cellular growth.[3d,6]



GSH = glutathione

Most studies on the growth inhibitory properties of α-ketoaldehydes have been carried out on bacteria and on mammalian cells, both normal and abnormal.[3,7] However, most enzyme studies on glyoxalase I, including those in our laboratory, have utilized the yeast enzyme.[8] We report here some comparative data on the growth inhibitory properties of α-ketoaldehydes toward bacteria and yeast. Specifically, we wished to determine whether the patterns of inhibition of growth of *Escherichia coli* K12 and *Saccharomyces cerevisiae* by a series of substituted phenylglyoxals show any correlation with the substrate specificity of glyoxalase I isolated from these organisms.

Experimental Section

Chemicals. Commercial methylglyoxal, 40% aqueous solution (Aldrich), was purified by distillation, and distillate was passed through an anion exchange resin (bicarbonate form) to remove lactic acid. The aromatic α-ketoaldehydes were prepared either by SeO_2 oxidation of the corresponding acetophenones[9] or by the procedure of Kornblum[10] involving conversion of substituted phenacyl nitrate esters into α-ketoaldehydes by treatment with sodium acetate in dimethyl sulfoxide. Characterization of these compounds has been reported.[10–12] Solutions of methylglyoxal were standardized enzymatically using glyoxalase I. Stock solutions of the aromatic α-ketoaldehydes (1:1 H_2O–ethanol) were prepared by weighing out appropriate amounts of the crystalline hydrates. Glutathione (Sigma) was standardized by titration with *N*-ethylmaleimide.[14] Deuterated analogs of methylglyoxal and phenylglyoxal were prepared as reported previously.[8c]

Purification of Glyoxalase I from *E. coli* K12. *E. coli* K12 was obtained as a frozen paste of cells harvested in mid-log phase (Grain Processing Corp.). Cells were fractured by homogenization in cold pH 7 phosphate buffer (μ = 0.2) with 10 m*M* magnesium chloride added, using glass beads and a Waring blender. Generally 50–100 g of paste was used. After centrifugation at 27,000 g for 30 min, the homogenate was fractionated with ammonium sulfate; activity precipitated in the 40–60% range. The precipitate was dissolved in pH 7 buffer, and the solution was chromatographed on CM-Sephadex (2.5 × 30 cm). Glyoxalase activity appeared as a single peak which was concentrated by microfiltration and purified further by gel chromatography on Sephadex G-100 (2 × 60 cm), giving a single peak of activity. *E. coli* glyoxalase I was unstable at all stages in this partial purification procedure but could be stabilized after the G-100 step by storing the concentrated material at −5° as a 50% glycerol solution. The material used in the kinetic studies was a 40-fold purification of the crude homogenate. Additional efforts to purify *E. coli* glyoxalase I are in progress. There is some evidence for a second form of the enzyme which does not survive the ammonium sulfate precipitation.

Yeast glyoxalase I (Sigma) was obtained as a 50% glycerol solution and either used directly or purified further.[8c] No evidence for more than one form of the yeast enzyme was observed.

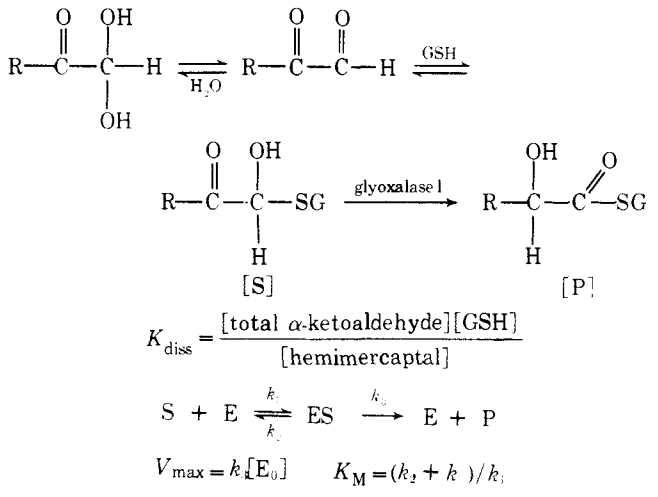
Kinetics. Kinetic studies of *E. coli* glyoxalase I and yeast glyoxalase I were carried out by following thiol ester formation at 240 nm for methylglyoxal[13] and by following the loss of reactant at the apparent isosbestic point between the α-ketoaldehyde and its glutathione adduct for the aromatic α-ketoaldehydes. The reaction conditions and spectral values have been reported except for 2,4,6-trimethylphenylglyoxal.[8b] This aromatic α-ketoaldehyde did not exhibit an isosbestic wavelength. The reaction was monitored at 270 nm where a fairly good approximation of an isosbestic point was observed, using extinction coefficients of 2050 M^{-1} cm^{-1} for the hemimercaptal and 670 M^{-1} cm^{-1} for the thiol ester product. The dissociation constant, K_{diss}, of the hemimercaptal was 0.94 m*M*. Kinetic data were treated by the Michaelis–Menten scheme; V_{max} and K_M values were obtained from double reciprocal plots,[15] assuming a one-substrate mechanism as shown in Scheme I.

Growth Studies. *E. coli* K12 cells were grown in peptone broth enriched with yeast extract and glucose, with shaking at 37°. Growth was followed by monitoring turbidity at 650 nm. Initially, growth was started in the absence of any inhibitor until the cells were in log phase. When growth reached an optical density of 0.2, 1-ml samples were taken and added to 20 ml of media containing 1 m*M* α-ketoaldehyde which was added from stock solutions of α-ketoaldehyde in dimethyl sulfoxide. The concentration of dimethyl sulfoxide in the growth flasks was 2–3%. Growth solutions without inhibitor but with 3% dimethyl sulfoxide were used as controls. The ability of an α-ketoaldehyde to inhibit cell growth was measured as the time lag before normal log growth resumed. The yeast studies were carried out similarly, except that the concentration of α-ketoaldehydes was 0.3 m*M*.

Results

The inhibition of growth of *E. coli* K12 in the presence of

Scheme I



$$K_{diss} = \frac{[\text{total } \alpha\text{-ketoaldehyde}][GSH]}{[\text{hemimercaptal}]}$$

$$S + E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P$$

$$V_{max} = k_3[E_0] \qquad K_M = (k_2 + k_3)/k_1$$

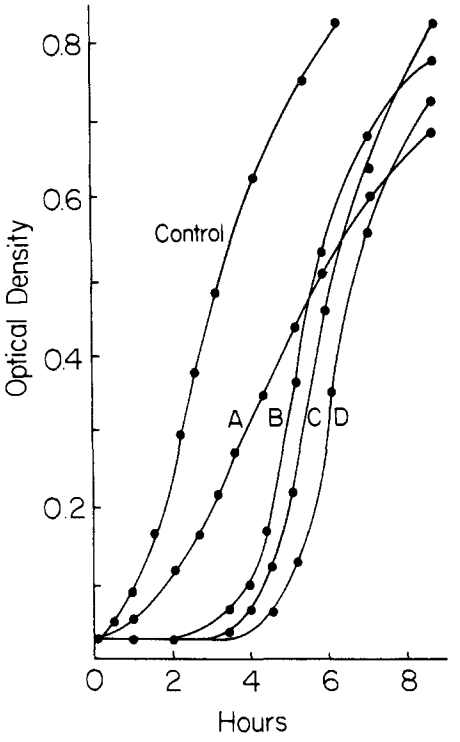


Figure 1. Inhibition of growth of *E. coli* K12 by α-ketoaldehydes; control is growth media with 3% dimethyl sulfoxide; A, B, C, and D are the control media with 1 m*M* concentrations of TMPG, MG, PG, and *p*-Cl-PG, respectively.

1 m*M* methylglyoxal (MG), phenylglyoxal (PG), *p*-chlorophenylglyoxal (*p*-Cl-PG), or 2,4,6-trimethylphenylglyoxal (TMPG) is shown in Figure 1.[2] Qualitatively, these α-ketoaldehydes are similar except for TMPG. This α-ketoaldehyde affected the rate of growth but showed a rather small initial inhibition of growth. The other α-ketoaldehydes inhibited the reestablishment of log growth by 3–4 hr, but subsequent growth was very similar to the control. The effects of 3% dimethyl sulfoxide appear to be minor. The inhibition of growth of yeast in the presence of 0.3 m*M* MG, PG, *p*-Cl-PG, or TMPG is shown in Figure 2. All four of the α-ketoaldehydes are qualitatively similar in the patterns of inhibition, including TMPG. The length of inhibition is 2–7 hr, *p*-Cl-PG being the most effective. It appears that both aliphatic and aromatic α-ketoaldehydes have the general ability to inhibit the growth of bacteria and yeast and that this is mainly inhibition of initiation of growth,

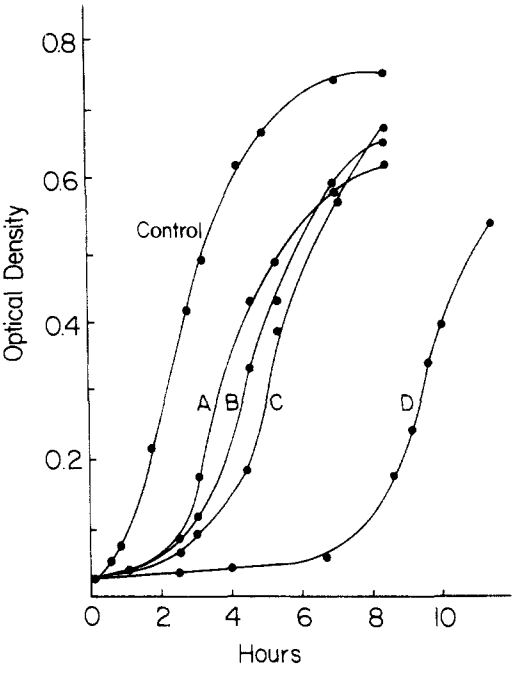


Figure 2. Inhibition of growth of yeast by α-ketoaldehydes; control is growth media with 3% dimethyl sulfoxide; A, B, C, and D are the control media with 0.3 m*M* concentrations of TMPG, MG, PG, and *p*-Cl-PG, respectively.

rather than an effect on the rate of growth once cell division begins. Other aromatic α-ketoaldehydes were also tested. All of them show patterns similar to the ones in Figures 1 and 2.

To test whether the period of growth inhibition represents the time required to produce higher levels of those enzymes involved in the metabolism of the α-ketoaldehydes, *E. coli* K12 inhibited with PG was allowed to resume normal growth and was then inhibited again with 1 m*M* PG. The period of inhibition was the same as in the first growth inhibition study. This could be repeated many times, suggesting that the period of growth inhibition represents the time required to metabolize the α-ketoaldehydes by enzymes present at the time the inhibitor is added. No evidence was found to suggest that higher concentrations of enzymes are produced, which should shorten subsequent periods of inhibition, nor was there any evidence of mutation, which might eliminate the sensitivity to α-ketoaldehydes.

One major question is the identity of the enzymes involved in the metabolism of α-ketoaldehydes during the period of growth inhibition. Previous studies on the substrate specificity of yeast glyoxalase I have shown that a very broad range of α-ketoaldehydes can be converted by glyoxalase I into the thiol esters of glutathione and α-hydroxycarboxylic acids.[8b] However, TMPG which has a sterically crowded side chain is not a substrate for the yeast enzyme.[16] Thus the patterns of inhibition shown in Figure 2 where TMPG is similar to the other α-ketoaldehydes suggest that the glyoxalase system may not be involved in detoxifying these inhibitors.

No data on the properties of glyoxalase I from a procaryote are available. Therefore the partial purification of glyoxalase I from *E. coli* K12 was carried out, and the kinetic parameters of the enzyme for the various α-ketoaldehydes were measured. The data are shown in Table I along with the kinetic parameters for yeast glyoxalase I, using the Michaelis–Menten treatment as shown in Scheme I. Whereas the yeast enzyme shows broad substrate speci-

Table I. Comparison of the Kinetic Parameters of Glyoxalase I from *E. coli* and Yeast[a]

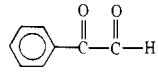
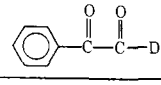
α-Ketoaldehyde	Yeast glyoxalase I		*E. coli* glyoxalase I	
	V_{max} (rel)[b]	K_M, M	V_{max} (rel)	K_M, M
Methylglyoxal	1.0	3×10^{-4}	1.0	4×10^{-5}
Phenylglyoxal	0.9	2×10^{-4}	133	2×10^{-4}
p-Chlorophenylglyoxal	0.5	4×10^{-5}	38	2×10^{-5}
2,4,6-Trimethylphenylglyoxal			9	1×10^{-3}

[a] pH 7, phosphate buffer, $\mu = 0.2$, with 10 m*M* $MgCl_2$ added; 25°. [b] V_{max} values are relative to methylglyoxal.

ficity for aliphatic and aromatic α-ketoaldehydes (except TMPG) reflected in V_{max} values which are insensitive to the nature of the α-ketoaldehyde, the enzyme from *E. coli* is quite different. The substrate specificity is even broader for the *E. coli* enzyme which acts on TMPG as well as the other α-ketoaldehydes. In addition, glyoxalase I from *E. coli* is very sensitive to the nature of the α-ketoaldehyde. Interestingly, the aromatic α-ketoaldehydes are considerably better substrates than MG, which is thought to be the normal substrate for the enzyme.

The markedly different kinetic properties of *E. coli* glyoxalase I and yeast glyoxalase I raised the question of whether there is a fundamental difference in reaction mechanism. The yeast enzyme catalyzes the disproportionation of the hemimercaptal of glutathione and an α-ketoaldehyde by a rate-determining intramolecular hydride migration.[8c] The *E. coli* enzyme was examined for deuterium isotope effects on V_{max}. The data are shown in Table II. The isotope effects are similar to those observed with yeast glyoxalase I, suggesting that the data in Table I are not the result of a fundamental change in reaction mechanism.

Table II. Deuterium Isotope Effects on the Glyoxalase I Catalyzed Disproportionation of the Glutathione Hemimercaptals of Methylglyoxal and Phenylglyoxal. Comparison of the *E. coli* and Yeast Enzymes[a]

α-Ketoaldehyde	Yeast glyoxalase I, $\frac{V_{max}(H)}{V_{max}(D)}$	*E. coli* glyoxalase I, $\frac{V_{max}(H)}{V_{max}(D)}$
$CH_3-C(=O)-C(=O)-H$	2.9	2.9
$CD_3-C(=O)-C(=O)-D$		
	2.4	3.2
		

[a] pH 7, phosphate buffer, $\mu = 0.2$, with 10 m*M* $MgCl_2$ added, 25°. $V_{max}(H)/V_{max}(D)$ is the ratio of the maximum velocities for the glyoxalase I reaction using a regular substrate and its deuterated analog.

Discussion

The comparative study of the kinetics of yeast and *E. coli* glyoxalase I toward a variety of α-ketoaldehydes and the abilities of these α-ketoaldehydes to inhibit the growth of bacteria and yeast raise two important points.

1. The kinetic parameters of these two enzymes are quite different. This suggests that the yeast enzyme also may be quite different from mammalian forms of glyoxalase I. Therefore one should be cautious in using the yeast enzyme to evaluate the potential usefulness of drugs designed to function as glyoxalase inhibitors.

2. The patterns of inhibition induced by aliphatic and aromatic α-ketoaldehydes on the growth of bacteria and yeast do not appear to implicate glyoxalase I as one of the main enzymes involved in removing the inhibitory effects of these compounds. This is based upon the observation that TMPG inhibits growth similar to the other aromatic α-ketoaldehydes even though it is not a substrate for yeast glyoxalase I. However, the possibility must be considered that the in vivo activity and specificity of the enzyme is different from that of the isolated enzyme. In addition, the apparent lack of correlation between the ability of various α-ketoaldehydes to inhibit cell growth and their substrate properties with glyoxalase I may be the result of rate-determining uptake of the α-ketoaldehydes. Consequently, the conclusion that glyoxalase I does not play a major role in detoxifying these compounds is only a tentative conclusion.

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References and Notes

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(2) Abbreviations: glutathione, GSH; methylglyoxal, MG; phenylglyoxal, PG; *p*-chlorophenylglyoxal, *p*-Cl-PG; 2,4,6-trimethylphenylglyoxal, TMPG.
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Synthesis and Antiinflammatory Activity of Some 2-Heteroaryl-α-methyl-5-benzoxazoleacetic Acids

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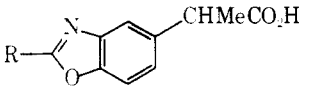
The syntheses of eight of the title compounds are described. The compounds possessed activity comparable with that of the previously tested 2-substituted phenyl analogs in the carrageenan-induced rat paw edema test.

2-(4-Chlorophenyl)-α-methyl-5-benzoxazoleacetic acid, benoxaprofen (Ia),[1] is a potent new antiinflammatory agent which is currently undergoing clinical trials. The antiinflammatory activities of some related 2-substituted phenylbenzoxazoles,[1] benzimidazoles,[2] benzothiazoles,[3] and benzothiazolines[3] have been reported recently.

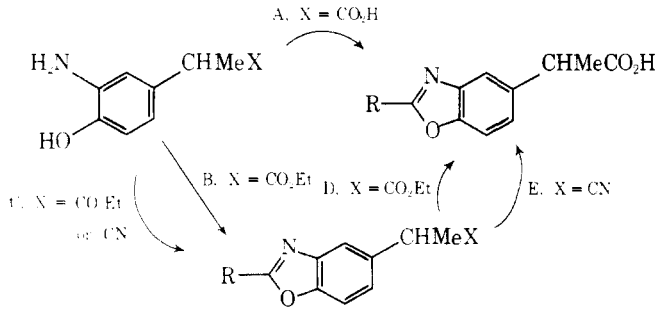


Ia, R = *p*-chlorophenyl
b, R = heteroaryl

The present communication describes the synthesis and antiinflammatory activity of a series of 2-heteroaryl-α-methyl-5-benzoxazoleacetic acids (Ib) which were investigated as part of a general program designed to define the structural requirements which were consistent with retention of activity in benoxaprofen analogs.

The new compounds are listed in Table I and they were prepared from 3-amino-4-hydroxy-α-methylbenzeneacetic acid, or the corresponding ester or nitrile,[1] by the methods outlined in Scheme I. Further details of the methods are given in the Experimental Section.

Scheme I. Synthesis of 2-Heteroaryl-α-methyl-5-benzoxazoleacetic Acids[a]



R = heteroaryl

[a]Main reagents for method A, RC(NH)OMe; B, RCOCl, then heat; C, RCHO, then $Pb(OAc)_4$; D, aqueous KOH or NaOH; E, concentrated HCl

The compounds were screened for antiinflammatory activity in the carrageenan-induced rat paw edema test.[1] Oral doses of the compounds were given to Wistar rats 3 and 0.5 hr before an injection of carrageenan and the amount of inflammation produced was compared with that formed in a control group of rats dosed with saline. The results are summarized in Table I, together with those for phenylbutazone which was tested concurrently as a control compound. The data for benoxaprofen are included for comparative purposes.

The heterocyclic derivatives 1, **2**, and 4–8 possessed significant activity at 50 mg/kg × 2 but none were superior to benoxaprofen on this test. The results also indicate that the 2-heteroarylbenzoxazoles possess activity of the same order as the previously tested 2-substituted phenyl analogs.[1]

Experimental Section

Elemental analyses were carried out by Mr. G. Maciak, Eli Lilly & Co., Indianapolis, Ind. Where analyses are indicated only by symbols of the elements, analytical results obtained for those elements were within ±0.4% of the theoretical values. The ir spectra were recorded on a Perkin-Elmer 457 spectrophotometer and the NMR spectra on a Varian A-60A spectrometer. The ir and NMR spectra for all of the analyzed compounds were consistent with the given structures. All of the prepared compounds are new.

α-Methyl-2-(2-pyridyl)-5-benzoxazoleacetic Acid (1). 2-Cyanopyridine (5.2 g, 0.05 mol) was added to a solution of sodium methoxide (0.005 mol) in MeOH (45 ml) and the solution was kept overnight at room temperature. AcOH (300 mg, 0.005 mol) was added, followed by 3-amino-4-hydroxy-α-methylbenzeneacetic acid[1] (9.05 g, 0.05 mol). The reaction mixture was stirred under reflux for 5 hr and evaporated under reduced pressure. The residue was dissolved in 2 *N* NaOH (150 ml) and extracted with Et_2O. The pH of the aqueous solution was adjusted to 6 with concentrated HCl. This yielded a solid which was filtered off and recrystallized from EtOH–H_2O. The dried cream crystals (7.5 g, 56%) had mp 177–179°. Anal. ($C_{15}H_{12}N_2O_3$) C, H, N.

α-Methyl-2-(3-pyridyl)-5-benzoxazoleacetic Acid (2). This was prepared in the same way as the foregoing product to give cream crystals (43%), mp 197–200°, from DMF–EtOH. Anal. ($C_{15}H_{12}N_2O_3$) C, H, N.

α-Methyl-2-(4-pyridyl)-5-benzoxazoleacetic Acid (3). This was prepared in the same way as compound 1. The cream crystals (60%), mp 247–250°, were obtained by recrystallization from DMF. Anal. ($C_{15}H_{12}N_2O_3$) C, H, N.

α-Methyl-2-(2-thienyl)-5-benzoxazoleacetic Acid (4). 2-Thenoyl chloride (9.9 g, 0.068 mol) was added carefully to a solution of ethyl 3-amino-4-hydroxy-α-methylbenzeneacetate[1] (12.9 g, 0.062 mol) in anhydrous pyridine (50 ml). This solution was heated at 100° for 3.5 hr and evaporated under reduced pressure. The residue was heated at 230° for 15 min while the vapor was allowed to escape. The cooled residue was then dissolved in EtOH (50 ml) to which a solution of KOH (10 g) in H_2O (10 ml) had been added previously. The reaction was stirred at room temperature for 19 hr and the expected product was isolated in the conventional manner. The cream crystals (7.5 g, 45%) had mp 161–163°. Anal. ($C_{14}H_{11}NO_3S$) C, H, N.

2-(5-Choro-2-thienyl)-α-methyl-5-benzoxazoleacetic Acid (5). A solution of ethyl 3-amino-4-hydroxy-α-methylbenzeneace-