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STUDIES ON ALDOSE REDUCTASE INHIBITORS FROM FUNGI. I. CITRININ AND RELATED BENZOPYRAN DERIVATIVES

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The fungal metabolites, citrinin (4,6-dihydro-8-hydroxy-3,4,5-trimethyl-6-oxo-3*H*-2-benzopyran-7-carboxylic acid) and DHMI (3,4-dihydro-6-methoxy-3,7-dimethyl-1*H*-2-benzopyran-8-ol), as well as certain synthetic derivatives, have been evaluated for aldose reductase inhibitory activity using a rat lens enzyme preparation. Citrinin and its reduction product, dihydrocitrinin, were found to have significant activity ($IC_{50} \approx 10\ \mu M$), whereas the other compounds were 3–10 times less potent. Kinetic studies showed that citrinin was not an irreversible inhibitor of the enzyme, as might be expected of a quinone methide. Spectroscopic (NMR) evidence is presented for the existence of citrinin predominantly in the form of its hemi-acetal in aqueous solutions, suggesting that it is this benzo[c]pyran derivative which interacts with the enzyme, rather than the quinone methide form.

KEY WORDS: Aldose reductase inhibitors, citrinin, DHMI, benzo[c]pyran derivatives.

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INTRODUCTION

There is a considerable amount of evidence implicating the enzyme aldose reductase (EC.1.1.1.21) in the pathogenesis of the complications of chronic diabetes.[1] Aldose reductase (AR), along with sorbitol dehydrogenase (SDH), form the polyol pathway which converts glucose to fructose in many mammalian tissues. In this pathway, AR catalyzes the NADPH-dependent reduction of glucose to sorbitol, while SDH uses NAD^+ to oxidize sorbitol to fructose. During the hyperglycemia associated with diabetes, there is an increased flux of glucose through the polyol pathway, and AR-catalyzed reduction to sorbitol. Since sorbitol is not efficiently oxidized by SDH, it accumulates intracellularly, and this accumulation is linked to a number of biochemical changes such as hyperosmosis, myoinositol depletion, and decreased activity of Na^+/K^+ ATPase. These biochemical changes ultimately are manifested as chronic diabetic pathologies, including cataracts, retinopathy, neuropathies and nephropathies. Since AR appears to initiate this sequence of events, this enzyme has become an attractive target for pharmacological intervention to prevent, or delay, the onset of diabetic complications. Various AR inhibitors (ARIs) have been discovered using *in vitro* models, and a number of these have been found to reverse the biochemical changes associated with chronic diabetes, and to mitigate the

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disease-associated pathologies.[2] The ARIs may be broadly sub-categorized as carboxylic acids, hydantoin derivatives, and phenolic natural products. The hydantoin and phenol ARIs are also acidic, and many contain a benzopyran moiety.

These observations prompted the present study to determine the AR-inhibitory potential of the fungal metabolite citrinin (4,6-dihydro-8-hydroxy-3,4,5-trimethyl-6-oxo-3*H*-2-benzopyran-7-carboxylic acid) (**1**) (Figure 1), and some related compounds. In common with many ARIs, citrinin possesses an acidic carboxyl group, a moderately acidic phenol-like (enol) functionality, and a ring-fused pyran system. Citrinin also contains a reactive quinone methide moiety, a functionality known to undergo facile nucleophilic attack.

Citrinin is produced by fungi belonging to the genera *Aspergillus*, *Penicillium*, *Pythium*[3] and *Cercosporidium*,[4] as well as by the flowering plant, *Crotalaria crispata*.[5] This metabolite exhibits a wide range of activities in diverse biological systems, including antimicrobial,[6] phytotoxic,[7] cytotoxic,[8] hypocholesterolemic,[9] and enzyme-inhibitory effects.[10] Natsume *et al.*[11] have suggested that the aberrant cell morphology induced by citrinin in the fungus *Cochliobolus lunatus* is due to the reaction of citrinin with a biological amine, and have reported solution spectroscopic data for the model methylamine adduct. However, quinone methides such as citrinin are known to be susceptible to attack by a wide range of nucleophiles, including –OH and –OR, with concomitant aromatization of the quinonoid ring.[12] Although such adducts tend to be unstable towards isolation, there is evidence for their presence in solution, and we surmised that, in dilute aqueous solution, citrinin would exist preferentially as the hemi-acetal. This hypothesis was supported by NMR-spectroscopic studies. However, since the hemi-acetal of citrinin is not isolable, we examined the structurally analogous dihydrocitrinin (3,4-dihydro-6,8-dihydroxy-3,4,5-trimethyl-1*H*-2-benzopyran-7-carboxylic acid) (**2**) (Figure 1), which differs from citrinin hemi-acetal only in the absence of a 1-OH group, for ARI-activity. In order to obtain further information regarding structure-activity relationships, we also tested another fungal metabolite, 3,4-dihydro-6-methoxy-3,7-dimethyl-1*H*-2-benzopyran-8-ol (DHMI) (**3**) (Figure 1), originally isolated from *Penicillium steckii* (NRRL 6336)[13] and *P. corylophilum* (ATCC 64543),[14] together with its synthetic dimethyl ether (3,4-dihydro-6,8-dimethoxy-3,7-dimethyl 1-*H*2-benzopyran) (**4**) (Figure 1) and *N*-methyl carbamate (3,4-dihydro-6-methoxy-3,7-dimethyl-1*H*-2-benzopyran-8-ol methyl carbamate) (**5**) (Figure 1) derivatives.

MATERIALS AND METHODS

Melting points were measured on a microscope hot-stage apparatus, and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on Bruker AC-250 and AM-400 spectrometers. Mass spectra were obtained using an Extel C-50/400 quadrupole spectrometer operating at 70 eV, with the ion source at 175°C. IR spectra were recorded with a Beckman 4210 spectrometer from thin films on KBr. Elemental analysis was performed by Atlantic Microlab, Norcross, Ga.

Isolation of Metabolites

Citrinin (**1**) was obtained from *Penicillium citrinum* NRRL 5907, according to the procedure described by Davis *et al.*[15] It was recrystallized from 95% ethanol to give

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lemon-yellow needles, whose physical and spectroscopic characteristics were consistent with those described in the literature.[16] DHMI (**3**) was obtained from *Penicillum corylophilum* ATCC 64543, as reported earlier.[13]

Synthesis of Derivatives

(*3,4-dihydro-6,8-dihydroxy-3,4,5-trimethyl-1*H-*2-benzopyran-7-carboxylic acid*) (**2**). To a stirred solution of (**1**) (32 mg; 0.13 mM) in EtOAc (2 ml), which had been sparged with N_2, was added PtO_2 (7 mg), and the mixture was then stirred overnight, at RT, under an atmosphere of H_2. The catalyst was removed by filtration of the mixture through a small quantity of Celite in a sintered-glass funnel, and the solvent evaporated under a stream of N_2, followed by high vacuum. The crude product was obtained as a glass (30 mg), homogeneous by TLC, which gave colourless crystals from benzene, m.p. 168–170° (lit.[17] m.p. 171°). ^{1}H NMR (250 MHz) ($CDCl_3$), δ 1.26 (6H, apparent d, J = 6.5 Hz, CH_3–9, CH_3–10), 2.10 (3H, s, H-11), 2.69 (1H, apparent q, H-4), 4.04 (1H, apparent q, H-3), 4.73 (2H, s, H-$1_{a'b}$), 8.3 (2H, v. br, –OH). ^{13}C NMR (63 MHz) ($CDCl_3$), ppm: 172.8, 157.3, 154.2, 146.5, 114.4, 111.7, 96.8, 74.0, 58.6, 35.7, 19.9, 17.9, 10.0.

(*3,4-dihydro-6,8-dimethoxy-3,7-dimethyl-1*H-*2-benzopyran* (**4**). A solution of (**3**) (62 mg; 0.30 mM) in dry DMF (4 ml) was added dropwise to a suspension of NaH (62 mg of 50% dispersion in oil; 1.3 mM) in dry DMF (4 ml). After the mixture had been stirred at RT for 15 min, iodomethane (0.5 ml; 1.14 g; 8.0 mM) was added to it dropwise. The reaction mixture was stirred at RT overnight, then poured into water (35 ml). The aqueous mixture was extracted with ether (4 × 20 ml), then the combined ethereal extracts washed successively with water (5 × 20 ml), 5% aqueous $Na_2S_2O_3$ (20 ml), and water (20 ml). The ethereal solution was dried over anhydrous Na_2SO_4 and filtered. Evaporation of the solvent yielded a semi-crystalline, solid residue. This crude product was chromatographed on silica gel (3 g), eluting with 30–60 petroleum ether (20 ml), then dichloromethane (20 ml). Evaporation of the fraction eluted with dichloromethane gave a solid (51 mg), which was recrystallized from methanol-water to give colourless crystals, m.p. 91–93° (lit.[13] m.p. 93–94°). ^{1}H NMR ($CDCl_3$), δ 1.37 (3H, d, J = 6.1 Hz, CH_3–3), 2.04 (3H, s, CH_3–7), 2.42 (1H, apparent dd, J = 11, 16 Hz, H-$4_{a'b}$), 2.63 (1H, apparent dd, J = 2, 17 Hz, H-$4_{a'b}$), 3.73 (1H, m, H-3), 3.80 (3H, s, CH_3–O), 3.83 (3H, s, CH_3–O), 4.58 (1H, d, J = 15.2 Hz, H-$1_{a'b}$) 6.34 (1H, s, H-5). ^{13}C NMR ($CDCl_3$), ppm: 156.4, 153.8, 134.0, 115.8, 115.4, 92.7, 70.4, 64.6, 55.8*, 55.2*, 34.0, 21.7, 10.0.

(*3,4-dihydro-6-methoxy-3,7-dimethyl-1*H-*2-benzopyran-8-ol methyl carbamate*) (**5**). Compound (**3**) (50 mg; 0.24 mM) was dissolved in acetone (2 ml), containing a trace of NEt_3 (prepared by adding 1 drop of NEt_3 to 10 ml acetone) and to the stirred solution was added methyl isocyanate (0.1 ml; 97 mg; 1.7 mM). The resulting homogeneous solution was allowed to stand overnight at RT. Volatiles were removed from the reaction mixture by evaporation under a stream of N_2, leaving a colorless, crystalline solid. This was recrystallized from ethyl acetate/hexane to give a product which was homogeneous by TLC, but which showed spurious peaks in its NMR

* Re-examination of the original spectra indicates that the shifts (57.90 and 57.20 ppm, respectively) reported in Reference 13 are in error.

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spectra. This material was purified further by means of a Chromatotron, on silica developed with 2:1 hexane:ethyl acetate, to produce colorless crystals (29 mg), m.p. 158–160°. ^{1}H NMR ($CDCl_3$), δ 1.36 (3H, d, J = 6.2 Hz, CH_3–3), 1.98 (3H, s, CH_3–7), 2.42 (1H, apparent dd, J = 10, 16 Hz, H-$4_{a'b}$), 2.60 (1H, apparent d, J = 16 Hz, H-$4_{a'b}$), 2.90 (3H, d, J = 4.8 Hz, CH_3–N), 3.72 (1H, m, H-3), 3.74 (3H, s, CH_3–O), 4.57 (1H, d, J = 15.8 Hz, H-$1_{a'b}$), 4.89 (1H, d, J = 15.8 Hz, H-$1_{a'b}$), 5.1 (1H, br), 6.44 (1H, s, H-5). ^{13}C NMR ($CDCl_3$), ppm: 155.3, 153.6, 147.8, 134.2, 120.9, 120.0, 102.1, 70.1, 64.6, 55.2, 33.8, 27.7, 21.6, 10.7. IR (thin film, KBr), cm^{-1}: 3300–3430 br, 1735, 1690, 1480, 1265, 1250, 1200, 1125, 1080. EI-MS: m/z 265 (18) (M^+), 208 (100) (M^+–CH_3NCO), 193 (31), 164 (63). Elemental analysis, found: C, 63.35; H, 7.18; N, 5.31. $C_{14}H_{19}NO_4$ requires: C, 63.38; H, 7.22; N, 5.28%.

Enzyme isolation and assay

Aldose reductase was isolated from the lenses of rat eyes as described earlier,[18] following the method of Kador *et al.*[19] Enzyme activity was assayed spectrophotometrically at 30°C by determining the decrease in NADPH absorbance at 340 nm in a Shimadzu UV-160 spectrophotometer equipped with a thermo-controlled multi-cell positioner. The control reaction mixture contained 0.104 mM NADPH (Sigma Type I) in 0.1 M phosphate buffer, pH 6.2; 10 mM D,L-glyceraldehyde (Sigma Chemical Co.), 0.2 ml of enzyme solution and distilled water in a total volume of 2.0 ml. A solvent blank was used and contained all of the above reagents, except glyceraldehyde, to correct for any oxidation of NADPH not associated with reduction of substrate. The reaction was initiated by the addition of substrate, and was monitored for 3 min following a 45 s initiation period. Enzyme activity was adjusted by dilution of the enzyme solution with distilled water, such that 0.2 ml of supernatant gave an average reaction rate for the control reaction of 0.0120 ± 0.0020 absorbance units/min.

Effects of Inhibitors

Inhibitory activities were determined by including 0.2 ml of an aqueous solution of the inhibitor at the desired concentration in the reaction mixture. Each inhibitor was tested at an initial concentration of 50 μM, and additional concentrations (40, 30, 20, 10, 5 μM, etc.) were employed to construct log concentration-percent inhibition curves. Each compound was tested at no fewer than four different concentrations, with a minimum of four determinations at each concentration. The percent inhibition for each inhibitor was calculated at all concentrations by comparing the rate of reactions containing inhibitor to that of control reactions with no inhibitor. Inhibitor IC_{50} values were then obtained by least squares analyses of the linear portion of the log inhibitor concentration versus percent inhibition curves using the LINEFIT program.[20]

Kinetic Studies

Kinetic studies were carried out using two to four different concentrations of each inhibitor. For substrate kinetics, the concentrations of the substrate D,L-glyceraldehyde ranged from 5.0 mM to 0.16 mM; for co-factor kinetics, the concentrations of NADPH were varied from 104 μM to 3.24 μM. The nature of the

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inhibition produced by each concentration of the inhibitor was determined by analysis of double reciprocal plots of enzyme velocity versus [D,L-glyceraldehyde] or [NADPH] as generated by least squares fit of the data using the program of Barlow.[20]

RESULTS AND DISCUSSION

The results from the rat lens assay are given in Table I; citrinin (**1**) and dihydrocitrinin (**2**) were the most inhibitory of the compounds tested. Citrinin (IC_{50} = 8.7 μM) and dihydrocitrinin were equi-active, approximately three times as inhibitory as DHMI (**3**) and its *N*-methyl carbamate (**5**), and more than ten times as potent as the dimethyl ether (**4**). As noted earlier, citrinin possesses a reactive quinone methide moiety and therefore may be capable of irreversibly inactivating enzymes such as AR. However, no evidence of irreversible inactivation of AR was detected in time-dependent inhibition studies. For example, no increase in AR inhibition was observed over a 30 minute period when the enzyme was incubated with 10 μM citrinin (approximate IC_{50} concentration). The inability of citrinin to irreversible inactivate AR may simply reflect the absence of a suitably positioned nucleophilic residue on the enzyme when citrinin is bound. Alternatively, it is possible that citrinin does not exist in the reactive quinone methide form under assay conditions, but rather is converted to the relatively unreactive hemi-acetal (**6**) as shown in Scheme 1. Support for this latter hypothesis was derived from UV and NMR-monitored reactivity studies on citrinin performed with various nucleophiles in organic and aqueous media. In $CDCl_3$, citrinin was found to react rapidly with amines (as noted originally by Natsume *et al.*[11]) and thiols to yield the corresponding acetals (Scheme 1). UV studies also indicated that citrinin reacts with water and slowly with ethanol, again presumably to form acetals. The reactivity of citrinin in aqueous media was analyzed in more detail by proton and carbon NMR; the compound was dissolved in D_2O containing one equivalent of $NaHCO_3$, giving a solution with pH ~8. The results of these studies, as summarized in Table II, strongly support the formation of citrinin hemi-acetal in water. Thus, the H-1 resonance of citrinin, which is a singlet at 8.21 ppm in $CDCl_3$, becomes two singlets, at 5.79 and 5.83 ppm, in D_2O, consistent with the expected chemical shift for hemi-acetal methines. A similar doubling of signals, and change in chemical shift, occurs for the other protons. Analogous changes in chemical

TABLE I
AR-Inhibitory of citrinin and analogues

Compound	IC_{50}, μM (95% CL)
1	8.7 (6.4–12)
2	12 (6.6–20)
3	33 (21–52)
4	100 (84–122)
5	35 (26–49)
sorbinil[a]	0.26 (0.15–0.36)

[a](S)(+)-6-fluoro-2,3-dihydrospiro-[4*H*-1-benzopyran-4,4′-imidazoline]-2′,5′-dione; standard ARI.

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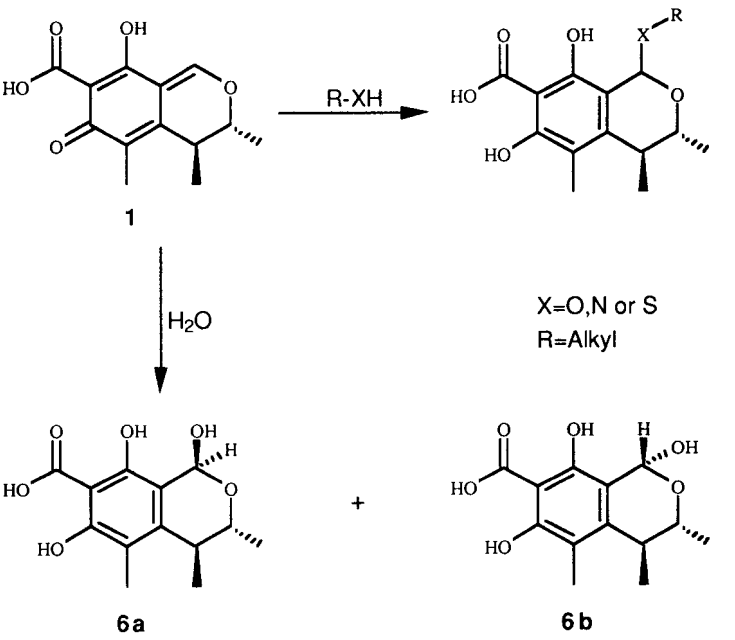

TABLE II
Partial ^1H and ^{13}C NMR data for citrinin in $CDCl_3$ and D_2O[a]

Position	δ ^{13}C ($CDCl_3$)[b]	δ ^{13}C (D_2O)[a]	δ ^1H ($CDCl_3$)	δ ^1H (D_2O)
1	162.9	73.5, 71.2	8.21 s	5.79 s, 5.83 s
3	81.8	87.4, 86.7	4.75 q	3.29 q, 3.98 q
4	34.5	36.2, 34.7	2.96 q	2.52 q, 2.64 q
9			1.33 d	1.14 d, 1.12 d
10			1.22 d	0.90 d, 1.01 d
11			2.00 s	1.82 s, 1.85 s

[a]Containing one equivalent of $NaHCO_3$ to facilitate dissolution.
[b]Data taken from Reference 21.

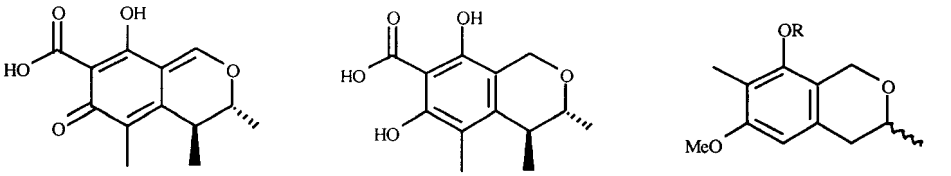

FIGURE 1 Structures of citrinin and analogues used in this study.

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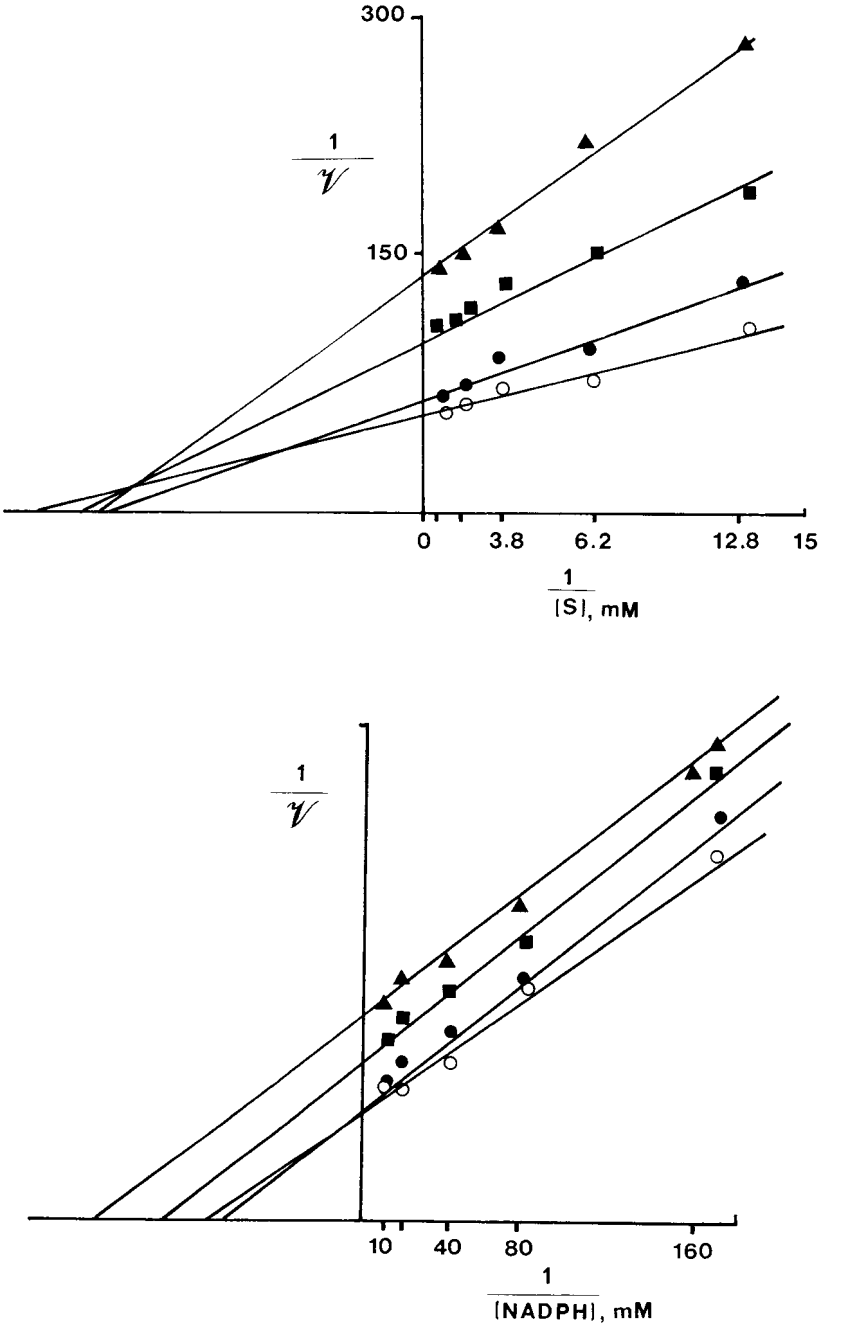

FIGURE 2 Lineweaver-Burk double reciprocal plots of initial enzyme velocity versus concentration of (a) the substrate glyceraldehyde [S] or (b) the cofactor NADPH in the presence of the inhibitor citrinin at 0 μM (○), 1.0 μM (●), 5.0 μM (■) and 10 μM (▲).

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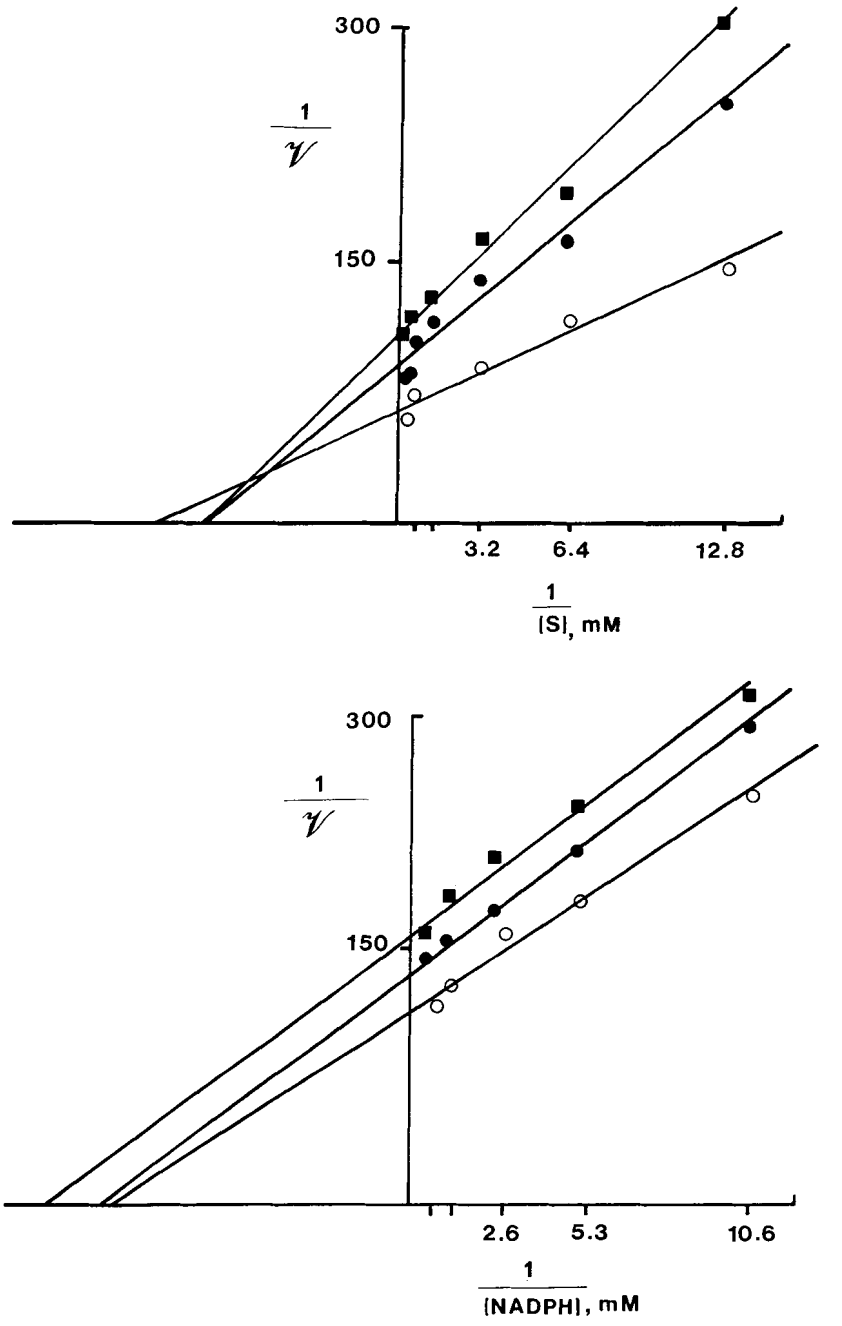

FIGURE 3 Lineweaver-Burk double reciprocal plots of initial enzyme velocity versus concentration of (a) the substrate glyceraldehyde [S] or (b) the cofactor NADPH in the presence of the inhibitor dihydrocitrinin at 0 μM (○), 5.0 μM (●), and 10 μM (■).

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shift, accompanied by a doubling of signals, are also evident in the ^{13}C-spectra, e.g. the vinylic C-1 resonance, at 162.9 ppm in $CDCl_3$, becomes two signals, at 73.5 and 71.2 ppm, in D_2O.

These data indicate that, when dissolved in water, citrinin rapidly forms a mixture of diastereomeric hemi-acetals, **6a** and **6b**, (Scheme 1), and suggest that these are the species actually studied under the conditions of a bioassay performed in an aqueous medium. The transformation of citrinin from an electrophilic quinone methide to a relatively unreactive hemi-acetal in water might also account for the absence of irreversible AR inhibition in our assay, as well as the similar inhibitory activities observed for citrinin and dihydrocitrinin. Under assay conditions, the only structural difference between these compounds is the hydroxyl group at C-1 in the hemi-acetal form of citrinin.

The lower inhibitory activities of DHMI (**3**), its *N*-methyl carbamate (**5**), and *O*-methyl ether (**4**) may result from several factors. None of these derivatives possesses a carboxylic acid moiety at C-7 and, based on the importance of this functionality in other series of ARIs, it is appealing to speculate that the absence of this group accounts for the lower ARI activity observed for these compounds. Also, comparing the activities of (**3**) and (**5**) suggests that the 8-hydroxy group of (**3**) does not contribute significantly towards inhibition since its conversion to a carbamate does not alter inhibitory activity (although it is possible that some hydrolysis of the carbamate back to the phenol may occur during the assay). On the other hand, when the 8-OH of (**3**) is methylated, as in the 8-OMe derivative, there is a three-fold diminution of activity, implying that only certain substitutions are tolerated at C-8.

Citrinin and its analogues also were evaluated to determine their kinetic profile of inhibition relative to both the substrate glyceraldehyde and the cofactor NADPH. For the most part, these compounds display mixed-type kinetics versus the substrate and uncompetitive versus the cofactor. This is illustrated by the kinetics of citrinin and dihydrocitrinin shown in Figures 2 and 3. There are subtle differences between the kinetics of these compounds, but these appear to result from the concentration of inhibitor rather than differences in kinetic mechanism. Furthermore, this type of kinetic behavior is common for inhibitors of AR and has been interpreted to mean that these compounds bind to a site distinct from the substrate and cofactor binding site, or bind at sites that only partially overlap those sites responsible for binding endogenous ligands. It should also be noted that the similarity in profiles for citrinin and dihydrocitrinin lend additional support for the hypothesis that citrinin exists in the hemi-acetal form under assay conditions.

Although the activity of citrinin has been evaluated in numerous biological systems, we have now shown that this microbial metabolite exhibits significant aldose reductase-inhibitory properties. In addition, we have demonstrated that, in aqueous biological systems, citrinin may exert its effects in the form of its hemi-acetal. Thus, the closely-related synthetic analogue, dihydrocitrinin, was found to have a potency comparable to that of citrinin in our assay. ARI activity, albeit of a lower order, was also noted in other compounds with structures based on the benzo[c]pyran skeleton, suggesting that this might be a useful new template for further synthetic elaboration and biological study.[21]

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22. Additional biological studies with dihydrocitrinin and analogues have been carried out, and will be published elsehwere.

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