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Synthesis, crystal structure and herbicidal activity of mimics of intermediates of the KARI reaction

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Abstract: Two mimics of the intermediate in the reaction catalyzed by ketol-acid reductoisomerase (KARI) were synthesized. Their structures were established on the basis of elemental analyses, IR, ^{1}H NMR and GC/mass detector. The crystal structure of compound 2 was found to be a substituted dioxane, formed by the condensation of two molecules. The two compounds showed some herbicidal activity on the basis of tests using rape root and barnyard grass growth inhibition. However, the herbicidal effect was weaker in greenhouse tests.

Keywords: KARI; metabolite mimics; crystal structure; herbicidal activity

1 INTRODUCTION

Plants have a more extensive biosynthetic capacity than animals, because the latter rely on their diet to supply many biochemical compounds and their precursors. Because of this difference, plants contain numerous enzymes that are potential targets for herbicides. The enzymes involved in the biosynthesis of the branched-chain amino acids leucine, isoleucine and valine are an example of such a pathway. Valine and isoleucine are synthesized in a parallel set of four reactions while leucine synthesis is an extension of the valine pathway.

The first successful herbicides to target this pathway were the sulfonylureas[1] and the imidazolinones,[2] both of which inhibit the first enzyme, acetohydroxyacid synthase (AHAS). Since the discovery of these herbicides a variety of other compounds targeting this enzyme have been found.[3] The success of these herbicides has stimulated research into inhibitors of other enzymes in the pathway, including two of those in the leucine branch[4,5] and the second enzyme in the common pathway, ketol-acid reductoisomerase (KARI). The reaction catalyzed by KARI is shown in Fig 1.[6]

The KARI reaction proceeds in two steps.[7,8] First, there is a magnesium-ion-dependent isomerization reaction that consists of an alkyl migration between carbon C2 and carbon C3 of the substrate and gives a methylhydroxyketol-acid. Second, the intermediate is transformed by an NADPH-dependent reduction of the ketone moiety to give the final product, and the reaction requires a divalent metal ion, such as Mg^{2+}, Mn^{2+} or Co^{2+}.

Inhibitors of KARI have been synthesized and tested as herbicides. HOE 704[9] and IpOHA[10] (Fig 2) are potent competitive inhibitors of the enzyme *in vitro*, but their activity as herbicides is weak. Dumas *et al*[11] suggested that this is due to their slow binding to the enzyme rather than KARI being an intrinsically poor herbicide target. Accumulation of the substrate *in vivo* would reverse the inhibition faster than it could develop, a phenomenon that has been described as 'metabolic resistance'.[12]

New inhibitors of KARI remain as a potential source of novel herbicidal compounds. With this in mind, we designed and synthesized two compounds (Fig 3; **1** and **2**) that mimic the structure of the β-hydroxy-α-keto-acid intermediate **A** shown as the bracketed structure in Fig 1. We have characterized their structures and assayed their herbicidal activity. The route of synthesis of the compounds is shown in Fig 3.

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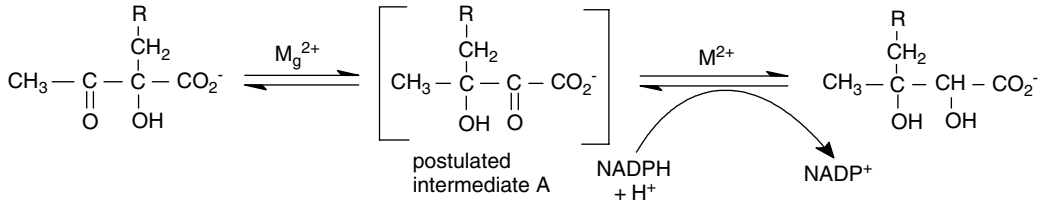

Figure 1. Reaction catalyzed by KARI.

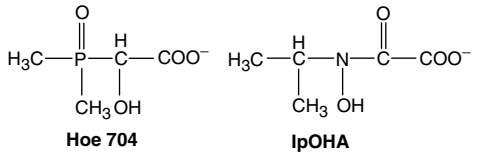

Figure 2. Structures of Hoe 704 and IpOHA.

2 EXPERIMENTAL

2.1 Methods and materials

Melting points were determined using a Yanaco MP-241 apparatus and are uncorrected. Infrared spectra were recorded on a Shimadzu IR-435 spectrophotometer as thin films or potassium bromide tablets. ^{1}H NMR spectra were measured on a Bruker AC-P500 instrument (300 MHz) using tetramethylsilane as an internal standard and deuterochloroform as solvent. Mass spectra were recorded on a Hewlett-Packard G1800A GC/mass detector instrument. Elemental analyses were performed on a Yanaco MT-3CHN elemental analyzer. The single crystal structure of compound **3** was determined on a Bruker SMART 1000 CCD diffractometer. Ethyl 3-methyl-2-oxobutanoate was purchased from Aldrich.

2.2 Syntheses

*2.2.1 Ethyl 3-methyl-3-hydroxy-2-oxobutanoate (**1**)*

The method is based on that described previously.[8] Ethyl 3-methyl-2-oxobutanoate (3.60 g, 0.025 mol) was placed in a 50-ml three-necked flask and a small amount of anhydrous hydrogen bromide passed through. The system was heated to 55 °C and then treated drop-wise with 1.25 ml (0.025 mol) of bromine while stirring. The red colour following the addition of one drop of bromine was allowed to dissipate before the next drop was added. After stirring at 55 ~ 60 °C for 2 h, during which time large amounts of hydrogen bromide gas were released, the mixture was cooled to room temperature to give a pale orange solution. The resulting crude ethyl 3-bromo-3-methyl-2-oxobutyrate was treated drop-wise with 3.45 g (0.025 mol) of potassium carbonate in a 150 g litre^{-1} aqueous solution and reacted for 1.5 h at 25 °C. The aqueous mixture was saturated with sodium chloride and extracted with ethyl acetate (5 × 15 ml). The organic layer was dried (anhydrous sodium sulfate) and the ethyl acetate removed under reduced pressure. The residual clear oil was distilled to give 3.10 g of compound **1** as a pale yellow liquid, bp 108 ~ 109 °C (27 mm Hg); ^{1}H NMR: δ: 4.33–4.40 (q, $J = 7.2$ Hz, 2H, CH_2), 3.13 (s, 1H, OH), 1.52 (s, 6H, $2CH_3$), 1.37–1.41 (t, $J = 7.2$ Hz, 3H, CH_3); IR, ν: 3514 (O–H), 2986, 2940 (CH_3,CH_2), 1732(C=O); MS, m/z: 160 (M^+); Anal: calcd for $C_7H_{12}O_4$; C 52.49, H 7.55; found C 52.41, H 7.50. The total yield of the two steps was 77.5%.

*2.2.2 Ethyl 3-hydroxy-2-oxobutanoate (**2**)*

Ethyl 2-oxobutanoate (5 g, 0.038 mol), prepared according to the literature[13] was placed in a 50-ml three-necked flask and a small amount of anhydrous hydrogen bromide passed through. The system was heated to 40 °C and then treated drop-wise with 2 ml (0.04 mole) of bromine while stirring. The red colour following the addition of one drop of bromine was allowed to dissipate before the next drop was added. After stirring at 40 ~ 45 °C for 2 h, the mixture was cooled to room temperature to give a pale orange solution. The resulting crude ethyl 3-bromo-2-oxobutyrate was treated drop-wise with a

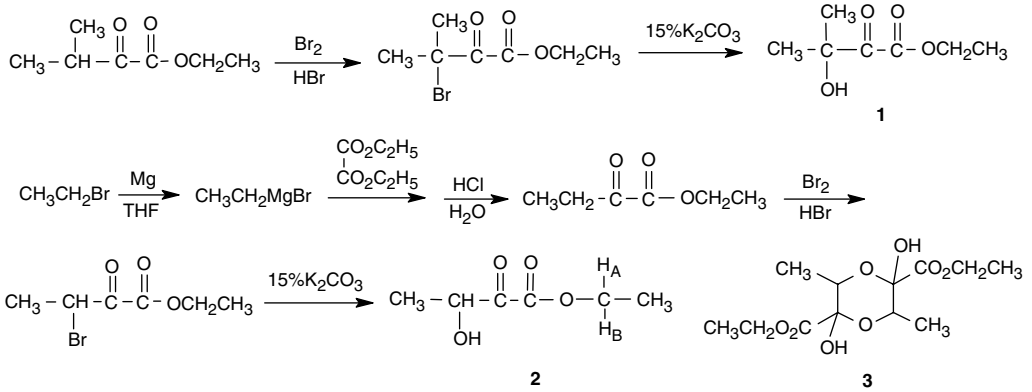

Figure 3. Synthetic route for compounds studied.

15% aqueous solution containing 5.52 g (0.04 mole) of potassium carbonate as a 150 g litre^{-1} aqueous solution and reacted for 1.5 h at 35 °C. The aqueous mixture was saturated with sodium chloride and extracted with ethyl acetate (5 × 20 ml). There was white layer between the organic layer and the water layer and this was combined with the ethyl acetate layer. After standing, a white solid appeared and was removed. The filtrate was then dried (anhydrous sodium sulfate) and the ethyl acetate removed under reduced pressure to give compound **2**. This material combined with the solid separated earlier had a total weight of 2.4 g, yield 43%; mp 127–129 °C; ^{1}H NMR; δ: 4.47–4.68 (q, $\mathcal{J} = 6.6$ Hz, 1H, CH), 4.20–4.31 (dq, $\mathcal{J}_{AB} = 10.8$ Hz, $\mathcal{J} = 7.0$ Hz, 1H, CH-H_A), 4.36–4.47 (dq, $\mathcal{J}_{AB} = 10.8$ Hz, $\mathcal{J} = 7.0$ Hz, 1H, CH-H_B), 4.01 (s, 1H, OH), 1.32–1.36 (t, $\mathcal{J} = 7.0$ Hz, 3H, CH_3), 1.09–1.11 (d, $\mathcal{J} = 6.6$ Hz, 3H, CH_3); IR, ν: 3486 (O-H), 2988, 2937 (CH_3, CH_2), 1735 (C=O); MS, m/z: 146 (M^+); Anal: calcd for $C_6H_{10}O_4$; C 49.31, H 6.90; found C 49.19, H 6.95.

2.3 Crystal structure determination

Compound **2** was dissolved in hot alcohol and diethyl ether, and the resulting colourless solution allowed to stand in air at room temperature to give single crystals of **3**. A colourless single crystal of **3** suitable for X-ray diffraction with dimensions of 0.24 × 0.24 × 0.18 mm was mounted on a Bruker SMART 1000 CCD diffractometer with Mo-Kα radiation ($\lambda = 0.71073$ Å) for data collection. A total of 2001 reflections were collected in the range of $2.57 < \theta < 26.44$ by the $\omega - 2\theta$ scan technique at 293(2) K, of which 1413 were independent with $R_{int} = 0.0188$. The empirical absorption correction was applied by the SADABS program. The structure was solved by direct methods (SHELXS-97) and refined by the full-matrix least-squares techniques on F^2. Most of the non-hydrogen atoms were located from an E-map, and the others, except the hydrogen atoms, were determined with successive difference Fourier syntheses. The compound crystallized in space group P-1 of the triclinic system with cell parameters: $a = 6.733(2)$ Å, $b = 6.980(3)$ Å, $c = 8.078(3)$ Å, $\alpha = 85.684(6)°$, $\beta = 78.637(6)°$, $\gamma = 69.305(6)°$, $V = 348.2(2)$ Å^3, $Z = 1$, $D_c = 1.394$ g cm^{-3}, $\mu = 0.118$ mm^{-1} and $F(000) = 156$. The final refinement converged at $R = 0.0373$, $wR = 0.0884$ for 1045 observed reflections with $I > 2\sigma(I)$, where $W = 1/[\sigma^2(F_O{}^2) + (0.0451P)^2 + 0.04P]$ with $P = (\max(F_O{}^2, 0) + 2F_c{}^2)/3$, $S = 1.021$, $(\Delta/\sigma)_{max} = 0.000$, $(\Delta\rho)_{max} = 0.211$ e/Å^3 and $(\Delta\rho)_{min} = -0.165$ e/Å^3.

2.4 Herbicidal activity tests

*2.4.1 Inhibition of the root-growth of rape (*Brassica campestris *L)*

The compounds to be tested were made into emulsions to aid dissolution. Rape seeds were soaked in distilled water for 4 h before being placed on a filter paper in a 6-cm Petri plate, to which 2 ml of inhibitor solution had been added in advance. Usually, 15 seeds were used on each plate. The plate was placed in a dark room and allowed to germinate for 65 h at 28 (±1) °C. The lengths of 10 rape roots selected from each plate were measured and the means were calculated. The percentage inhibition was calculated relative to controls using distilled water instead of the inhibitor solution.

*2.4.2 Inhibition of the seedling growth of barnyard grass (*Echinochloa crus-galli *(L) Beauv)*

The compounds to be evaluated were made into emulsions to aid dissolution. Ten *E crus-galli* seeds were placed into a 50-ml cup covered with a layer of glass beads and a piece of filter paper at the bottom, to which 5 ml of inhibitor solution had been added in advance. The cup was placed in a bright room and the seeds allowed to germinate for 65 h at 28 (±1) °C. The heights of the above-ground parts of the seedlings in each cup were measured and the means calculated. The percentage inhibition was calculated relative to controls using distilled water instead of the inhibitor solution.

2.4.3 Glasshouse tests

2.4.3.1 Pre-emergence. Sandy clay (100 g) in a plastic box (11 × 7.5 × 6 cm) was wetted with water. Sprouting seeds (15) of the weed under test were planted in fine earth (0.6 cm depth) in the glasshouse and sprayed with the test compound dissolved in a suitable solvent at 1500 g ha^{-1}.

2.4.3.2 Post-emergence. Seedlings (one leaf and one stem) of the weed were sprayed with the test compounds at the same rate as used for the pre-emergence test.

For both methods, the fresh weights were determined 15 days later, and the percentage inhibition relative to water-sprayed controls was calculated.

3 RESULTS AND DISCUSSION

3.1 Synthesis

The ethyl 3-hydroxy-2-oxo-(substituted)-butanoate products can be synthesized by bromination and hydrolysis of the corresponding α-keto esters. Several methods reported for the synthesis of α-keto esters are based on the alcoholysis of acyl cyanides,[14] the addition of alkyl lithium reagents to triethoxyacetonitrile[15] and the peracid oxidation of diazoesters.[16] Weinstock *et al*[13] reported a one-pot synthesis of α-keto esters using diethyl oxalate and Grignard reagent. We preferred Weinstock's method to prepare ethyl 2-oxobutanoate for its less expensive reagents and greater convenience.

3.2 Spectroscopic properties

The IR spectra of the compounds tested shows absorption bands at 3500 cm^{-1} originating from the stretching vibration of O–H. The strong band at

1735 cm^{-1} can be assigned to the C=O stretching vibration. In the ^1H NMR spectrum of compound **2**, the methylene proton of ethoxyl was split into two octets of peaks instead of quadruple peaks by coupling with the adjacent methyl. However, the spectrum was normal for compound **1**. We suggest that the asymmetry of the molecular structure of **2** and the surrounding influence on CH_2 leads to the splitting of H_A and H_B. These protons are on the same carbon atom, but may be affected differently by the chiral carbon atom in the molecule. The two protons are split into double peaks that are affected by each other with $\mathcal{J} = 10.8$ Hz.

3.3 Crystal structure

Because of the unusual ^1H NMR spectrum of **2**, we investigated this compound further by determining its crystal structure. This analysis revealed that compound **3** was present. Possibly this is formed after standing in solvent for several weeks by condensation of two molecules of **2** (Fig 4a). The β-hydroxy and α-carbonyl of one molecule of **2** reacted simultaneously with the corresponding α-carbonyl and β-hydroxy of another molecule of **2** to give two hemiketals, thus forming a dioxane structure. The similar dimerization of glycolaldehyde is well established. In the dioxane six-membered ring, the ethoxycarbonyl and methyl are in the a-bond position of the chair conformation and the hydroxyls are in the e-bond position. From the cell packing diagram (Fig 4b) of compound **3**, it can be seen that there are intermolecular hydrogen bonds between H(3) and O(2) of two molecules, with bond lengths of 2.128 Å for H(3)–O(2) and 2.278 Å for O(2)–H(3). An eight-membered ring is formed through hydrogen bonds from the hydroxyl and ethoxycarbonyl of two dioxane structures. All atomic coordinates and equivalent isotropic displacement parameters are listed in Table 1, and selected bond lengths and bond angles are shown in Table 2.

3.4 Herbicidal activity of compounds

Compound **1** is the ethyl ester of the normal intermediate in valine and leucine synthesis. Analysis of the three-dimensional structure of spinach KARI[17]

Table 1. Atomic coordinates ($\times 10^4$) and equivalent isotropic displacement parameters (Å$^2 \times 10^3$) of compound **3**

Atom	*X*	*Y*	*Z*	U_{eq}[a]
O(1)	2860(2)	859(2)	1784(1)	39(1)
O(2)	1734(2)	2768(2)	4101(1)	50(1)
O(3)	1353(2)	5603(2)	2691(1)	38(1)
O(4)	1751(2)	5032(2)	641(1)	33(1)
C(1)	3134(4)	−2074(3)	3658(3)	59(1)
C(2)	4311(3)	−828(2)	2631(2)	42(1)
C(3)	1697(2)	2514(2)	2662(2)	31(1)
C(4)	267(2)	4177(2)	1625(2)	30(1)
C(5)	752(2)	6657(2)	−454(2)	31(1)
C(6)	2465(3)	7469(3)	−1357(2)	46(1)

[a] $U_{eq} = (1/3)\Sigma_i \Sigma_j U_{ij} \alpha_i * \alpha_j * \alpha_i \alpha_j$.

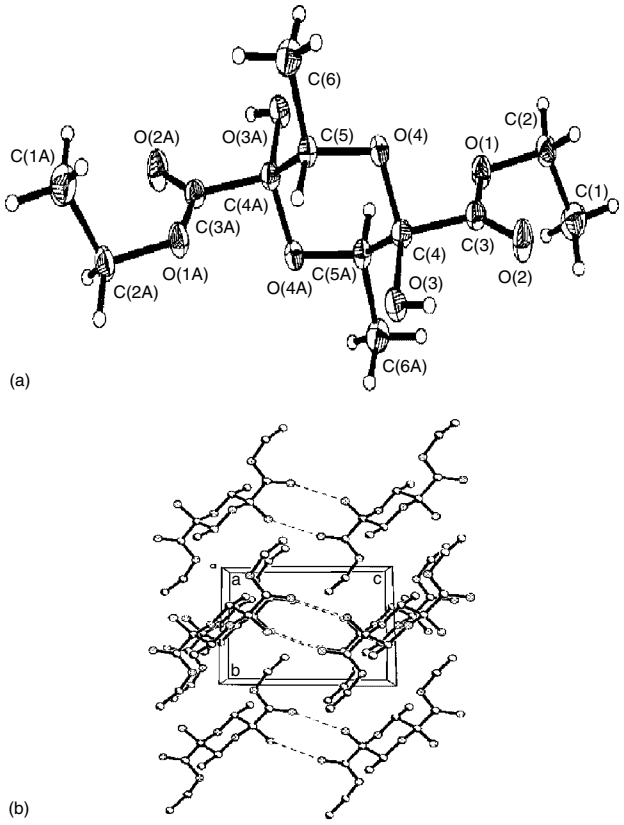

Figure 4. (a) Molecular structure of compound **3** and (b) its packing diagram in the unit cell.

Table 2. Selected bond lengths (Å) and bond angles (°) for compound **3**[a]

Bond length		Bond angle	
O(1)–C(3)	1.3104(18)	C(3)–O(1)–C(2)	117.59(12)
O(1)–C(2)	1.4627(18)	C(4)–O(4)–C(5)	112.73(11)
O(2)–C(3)	1.1953(18)	O(1)–C(2)–C(1)	111.15(15)
O(3)–C(4)	1.3832(17)	O(2)–C(3)–O(1)	125.33(14)
O(3)–H(3)	0.8200	O(2)–C(3)–C(4)	122.12(13)
O(4)–C(4)	1.4226(18)	O(1)–C(3)–C(4)	112.53(12)
O(4)–C(5)	1.4376(17)	O(3)–C(4)–O(4)	112.08(12)
C(1)–C(2)	1.481(3)	O(3)–C(4)–C(5)#1	108.92(13)
C(3)–C(4)	1.535(2)	O(4)–C(4)–C(5)#1	109.31(12)
C(4)–C(5)#	11.525(2)	O(3)–C(4)–C(3)	110.04(12)
C(5)–C(6)	1.501(2)	O(4)–C(4)–C(3)	102.75(12)
C(5)–C(4)#	11.525(2)	C(5)#1–C(4)–C(3)	113.69(12)
		O(4)–C(5)–C(6)	107.13(13)
		O(4)–C(5)–C(4)#1	108.77(12)
		C(6)–C(5)–C(4)#1	113.99(13)

[a] Symmetry code: #1 $-x, -y+1, -z$.

suggests that this compound could fit into the active site of the enzyme with the ethyl group protruding towards the solvent. The smaller compound **2** could also fit into the active site. In this way they could probably inhibit KARI and possess herbicidal activity. Consequently, the herbicidal activities of compounds **1** and **2** were tested and the results are shown in Table 3. Both compounds showed some herbicidal activity in the rape root and barnyard grass cup tests and compound **1** showed 82.8%

Table 3. Herbicidal activity of compounds (% inhibition)

	Rape root test			Barnyardgrass cup test	
Compound	100 μg ml^{-1}	10 μg ml^{-1}	1 μg ml^{-1}	100 μg ml^{-1}	10 μg ml^{-1}
1	82.8	23.6	—	34.3	13.9
2	9.1	2.6	—	24.4	12.8
Chlorsulfuron	—	—	64.2	—	—
Metsulfuron-methyl	—	—	81.0	—	—

Table 4. Herbicidal activity of compounds (% inhibition)[a]

	Echinochloa crus-galli		*Digitaria adscendens*		*Brassica campestris*		*Amaranthus retroflexus*		Lucerne	
Compound[b]	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre
1	14.9	0	27.9	0	0	0	17.9	0	6.8	0
2	9.4	11.7	21.3	14.5	0	0	0	0	0	6.7
4	18.2	5.1	42.6	17.4	25.8	0	31.1	14.3	34.6	10.0
5	15.6	3.0	25.2	0	5.9	0	11.5	0	13.6	13.3
Chlorsulfuron	100	68.0	30.0	0	100	98.0	100	92.6	100	81.0
Metsulfuron-methyl	100	88.2	100	89.0	100	98.1	100	81.0	100	92.0

[a] Post: post-emergence; Pre: pre-emergence.
[b] Rates: **1**, **2**, **4**, **5** 1500 g ha^{-1}, chlorsulfuron 30 g ha^{-1}, metsulfuron-methyl 15 g ha^{-1}.

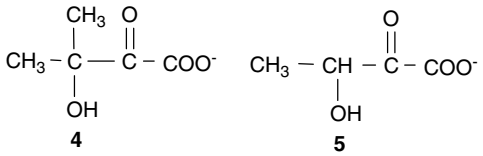

Figure 5. Structures of compounds **4** and **5**.

inhibition of rape root growth at a concentration of 100 μg ml^{-1}. In addition, the herbicidal activity of these compounds was bioassayed in the glasshouse on five herbs representative of monocotyledonous and dicotyledonous plants. The ethyl esters were further hydrolyzed into the carboxylic form (compounds **4** and **5** in Fig 5) using aqueous potassium hydroxide[8] and their herbicidal activities were also tested. The results of the greenhouse tests are shown in Table 4.

In comparison with two commercial sulfonylureas that were also tested, the compounds described here have quite low herbicidal activity. The results from the greenhouse tests showed that all of our compounds have comparatively weak herbicidal activity, but compound **1** shows clear inhibitory activity upon rape root growth. It is interesting to note that the hydrolyzed compounds **4** and **5** showed somewhat better activity than the unhydrolyzed esters. Compound **4** is supposed to be the true intermediate A in the KARI reaction, as shown in Fig 1, and compound **5** is a simplified analog of this intermediate. These results provide some interesting clues for further study of structure–activity relationship in KARI inhibitors.

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