Syntheses and Iron(II) Induced Reactions of Phenyl-Substituted 1,2,4-Trioxanes

Chang Ho Oh*[†] and Gary H. Posner[‡]

[†]*Department of Chemistry, Inje University, Kimhae 621-749, Korea*
[‡]*Department of Chemistry, The Johns Hopkins University, Baltimore, MD 21218, USA*
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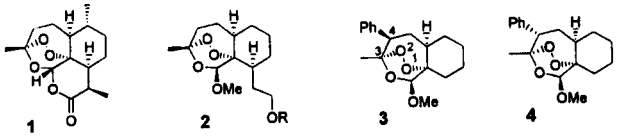
Introduction of an alkyl substituent at the $C_{4\beta}$ position of antimalarial trioxanes has caused them to become more active in their antimalarial activity. We have designed a structurally simple 4β-phenyl substituted trioxane (**3**) as an active antimalarial since it can form a more stable carbon radical when reacting with ferrous bromide. The trioxane **3** has been prepared along with the corresponding isomer **4** according to the previously reported procedure. The synthesized trioxanes **3** and **4** were finally separated by using HPLC and assigned their stereochemistry by spectroscopy and X-ray crystallography. Their antimalarial activities were surprisingly low. The low activity was then rationalized based on the product distribution of the ferrous ion induced reaction of these trioxanes. These trioxanes with ferrous bromide did not produce any detectable amount of the corresponding C_4-hydroxylated product, consistent with the fact that neither $C_{4\beta}$-phenyl substituted nor $C_{4\alpha}$-phenyl substituted trioxane has any antimalarial activity. It implies that a C_4 substituent of antimalarial trioxanes has to stabilize an adjacent carbon-centered radical in a specific stability range in order to show a good antimalarial activity. This study, combined with related studies, could help develop more potent antimalarial trioxanes.

Introduction

The resurgence of malaria as a worldwide health threat due to the causative parasites' rapidly increasing resistance to traditional alkaloidal drugs has prompted extensive investigation into alternative chemotheraphies. An extremely promising prospect from these searches is the sesquiterpene trioxane endoperoxide artemisinin (**1**).[1] Artemisinin, isolated from the Chinese herbal medicine Qinghao (*Artemisia annua* L.), is clinically used for treatment of malaria worldwide. The outstanding antimalarial activity, the novel structure, and low natural supply of artemisinin (Quinghaosu) have prompted extensive efforts to synthesize artemisinin and its analogs and model studies aimed at securing routes to the biologically crucial 1,2,4-trioxanes.[2]

The complete synthesis of artemisinin has been achieved by several groups after Schmid and coworkers reported the first total synthesis starting from (-)-isopulegol in 13 steps.[3] Given the synthesis of artemisinin and its semi-synthetic derivatives, recent efforts have been focused on the synthesis of structurally simple 1,2,4-trioxanes.[4] Structure-activity analysis reveals that the minimum requirements for antimalarial activity are the 1,2,4-trioxane ring and a second ring. Several synthetic trioxanes (**2**), synthesized by us, were shown to be as active as arteether, the ethyl ether of dihydroartemisinin, against multi-drug resistant *Plasmodium falciparum* in Aotus monkeys.[5]

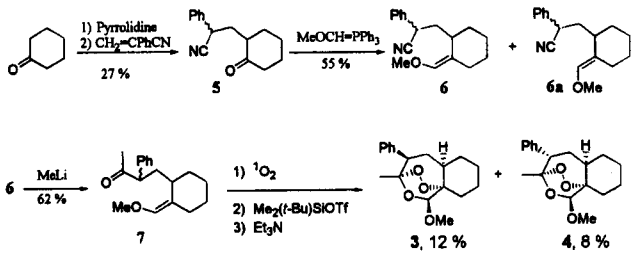


The stability and reactivity of a peroxide 1,2,4-trioxane moiety in artemisinin and its related analogs have been of much interest to many researchers. A number of reactions involving a 1,2,4-trioxane ring have been known such as hydrolysis, hydrogenation, reduction, and so on.[6] One of the most interesting among these is a ferrous ion induced reaction since iron in hemin reacts with trioxanes to form the relatively stable carbon radical which is believed to play a key role in its antimalarial activity.[7] Malaria-infected red cells are known to be very oxidant sensitive and even to increase lipid peroxidation. Many oxidant drugs are known to selectively damage parasite-infected red cells.[8] Malaria parasites get essential amino acids from the digestion of the host cell's hemoglobin and release large amounts of heme, a soluble iron porphyrin.[9] These hemins are rapidly polymerized to insoluble hemozoin, large granules of precipitated hemin. Parasites, if lacking hemozoin, appear to be insensitive to trioxane antimalarials.

In our previous studies, we have shown that ferrous ion reduces the crucial peroxide linkage to form oxy radical and then carbon radical intermediates leading to the C_4-hydroxylated product and the ring contracted product; of these two pathways, only the first involving a C_4 radical intermediate leading to the C_4-hydroxylated product **2a** is important for high antimalarial activity.[7] Meshnick and his coworkers have extensively studied the reaction between hemin and artemisinin under physiological conditions, and isolated a hemin-artemisinin adduct.[10] SAR study of several trioxanes bearing diverse substituents at C_4 has given significant and strong evidence supporting the key role and the limitations of such C_4 radicals in the antimalarial activity.[11] $C_{4\beta}$-phenyl substituted trioxane was expected to form a stable C_4 radical upon exposure to ferrous ion. Herein we report a full account for syntheses, antimalarial activities, and reactions toward ferrous bromide of both isomers of such interesting C_4-phenyl trioxanes.

Results and Discussion

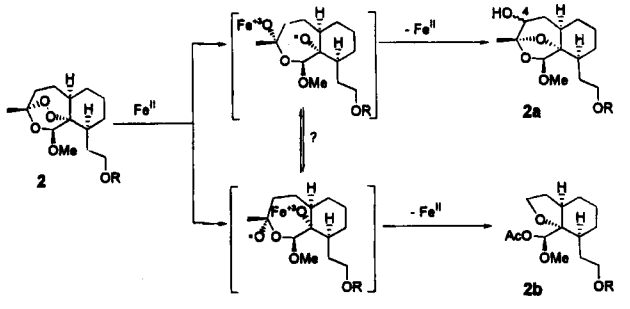
Synthesis of a pair of phenyl substituted 1,2,4-trioxanes. Phenyl substituted 1,2,4-trioxanes **3** and **4**



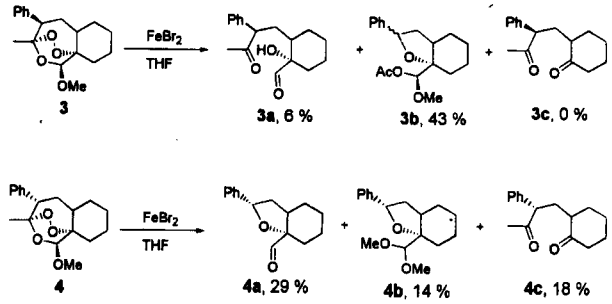
Scheme 1.

were synthesized as shown in Scheme 1. Phenyl acrylonitrile was, *in situ*, prepared by refluxing benzyl cyanide and paraformaldehyde in benzene in the presence of catalytic amount of sodium hydride. Refluxing benzene solution of phenylacrylonitrile and pyrrolidine enamine of cyclohexanone and then hydrolysis with 10% sulfuric acid afforded the desired product **5** as a mixture of diastereomers.[12] Since we do not know the relative stereochemistry of each of these isomers, we isolated both isomers for the next reactions. Methoxymethylenation to the enol ether **6** was performed as usual.[5a] Chromatographic separation afforded a mixture of three isomeric products **6** and a single isomer having *E*-configuration **6a**. Methylation of the product mixture **6** yielded the ketoenol ether **7** as isomeric mixtures. Silica gel chromatography afforded the *Z*-keto enolether **7** as a two diastereomeric mixtures and a *E*-keto enolether (**7a**). Photooxygenation of the keto enol ether **7** and then isomerization of the intermediate dioxetane using *tert*-butyldimethylsilyl triflate smoothly occur to give a mixture of the corresponding four 1,2,4-trioxanes.[11] Silica gel chromatography was used to separate the two major isomers **3** and **4** from the minor isomers. The major isomers were then separated by using normal phase HPLC to give **3** and **4** in 12% and 8% based on the substrate **7**, respectively. Spectroscopic data and X-ray study revealed that the both isomers have the assigned stereochemistry. Although the overall synthesis was not efficient from the synthetic viewpoint, our primary goal, the synthesis of 4β-phenylsubstituted trioxane, was achieved successfully.

Iron(II) Induced Reaction of Trioxanes 3 and 4. We have reported that iron(II) catalyzed an 1,2,4-trioxane to form a radical anion, an oxy radical and an alkoxide shown in Scheme 2. The reactive oxy radical can abstract hydrogen *via* 1,5 hydrogen shift to form the relatively stable carbon radical which is believed to play a key role in its antimalarial activity.[7] In fact, $C_{4\beta}$-alkyl substituted trioxanes



Scheme 2.



Scheme 3.

have shown to be more antimalarially active than the corresponding unsubstituted trioxanes. Likewise, $C_{4\alpha}$-alkyl substituted trioxanes were found to be almost inactive.[13]

Based on SAR data, we have postulated $C_{4\beta}$-phenyl trioxane to be highly potent, since it can form a stable carbon radical upon exposure to ferrous ion. Unexpectedly, $C_{4\beta}$ and $C_{4\alpha}$-phenyl substituted trioxanes were found to have no antimalarial activities *in vitro*, although the incorporation of a $C_{4\beta}$-phenyl substituent substantially stabilizes an adjacent carbon radical more effectively than a methyl or benzyl group.[11] The surprisingly low antimalarial activity of such $C_{4\beta}$ analogs prompted study of the product distribution upon exposure of both analogs **3** and **4** to ferrous ions (Scheme 3).

In contrast to the antimalarial benzyl ether **2** of the $C_{4\beta}$-methyl analog that reacted with ferrous bromide in THF to give a 1 : 4 ratio of a C_4-hydroxylated product like **2a** and a ring contracted product like **2b**, the $C_{4\beta}$-phenyl analog **3** reacted under similar conditions to form ring contracted acetal **3b** as the only major product; no more than a trace of any C_4-hydroxylated product like **2a** was detected. This result suggests that a $C_{4\beta}$-substituent that would make an adjacent carbon radical more stable in the upper pathway seems to shunt the ferrous ion reduction of that analog toward the lower pathway in Scheme 2, thereby actually avoiding formation of the C_4 radical intermediate that would lead to a C_4-hydroxylated product like **2a**, characteristic of a potent antimalarial trioxane. As expected, trioxane **4** having $C_{4\alpha}$-phenylsubstituted trioxane has been shown to have no antimalarial activity and its reaction with ferrous bromide did not produce any C_4-hydroxylated product like **2a**. Interestingly and unexpectedly, trioxane **4** did form different types of degradation products.

We carefully characterized the products derived from the reaction of the trioxane **3** and **4** with ferrous bromide. Trioxane **3** yielded the ring contracted product **3b** as a major product along with a structurally unknown product **3a**. In contrast, trioxane **4** yielded the ring contracted products **4a** and **4b**, and the fragmented diketone **4c** as the other major product.[14] Although both isomers toward ferrous bromide formed the corresponding ring-contracted products, they have shown some different behavior: while trioxane **4** formed the diketone **4c**, trioxane **3** did not form the diketone **3c**. Although we do not know the unambiguous chemical mechanism for these reactions, it is worthwhile to note that this study provide an opportunity for further chemical elaboration from the view point that 1,2,4-trioxanes have been virtually unexplored owing to their limited availability and the belief that they might be unstable and explosive.

Conclusion

We have designed a structurally simple 4β-phenyl substituted trioxane as a potent antimalarial candidate. The trioxane has been prepared along with the corresponding isomers according to the previously reported procedure. The low activity was then rationalized based on the product distribution of the ferrous ion induced reaction of this trioxane. Although $C_{4\beta}$-phenyl analog **3** could form an electronically stable carbon centered radical at the C-4 position, the bulkiness of the phenyl group with its neighboring groups might prevent the analog **3** from forming such a radical. In fact, the antimalarial activity was suprisingly low. It implies that a C_4 substituent of antimalarial trioxane has to stabilize an adjacent carbon-centered radical in a specific stability range in order to show a good antimalarial activity. This study, combined with related studies, could help develop more potent antimalaiarl trioxanes.

Experimental

Preparation of 3-(2-Oxocyclohexyl)-2-phenylpropanenitrile (5). In a 100 mL round bottomed flask were placed pyrrolidine (6.9 mL, 83 mmol), cyclohexanone (7.38 g, 75 mmol), and benzene (50 mL). Refluxing the solution with distilling off water over 3h formed 1-pyrrolidino-1-cyclohexene (*ca* 75 mmol). To the other 250 mL round-bottomed flask were charged paraformaldehyde (2.42 g, 80 mmol monomer), sodium hydride (60% dispersion on mineral oil, *ca* 150 mg, 3.8 mmol) and benzene (20 mL), and was treated benzylcyanide (5.86 g, 50 mmol) in benzene (30 mL) under argon atmosphere. The mixture was treated with the above enamine solution and refluxed overnight with distilling off water using a dean-stark trap. The final solution was cooled to room temperature, treated with water (30 mL) and 10% sulfuric acid solution (30 mL). The organic products was extracted with ether (150 mL × 3), combined, washed twice with saturated NaCl solution (100 mL × 2), dried over magnesium sulfate, and filtered. The filterate was concentrated under reduced pressure to give the crude products which mainly contained the desired products and the starting materials in about 1 : 2 ratio. Silica gel chromatography of the crude products gave the corresponding products (3.05 g, 27%) as a mixture of two diastereomers. Each isomer was further separated for spectral data. **5a** (nonpolar): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.46-7.27 (m, 5H), 4.16 (dd, *J*=11.3, 4.7 Hz, 1H), 2.70 (m, 1H), 2.50-2.35 (m, 2H), 2.30-2.20 (m, 1H), 2.20-2.10 (m, 2H), 1.95-1.56 (m, 4H), 1.47-1.35 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 212.21, 136.25, 129.02, 127.94, 126.99, 120.95, 48.33, 42.36, 37.11, 35.76, 35.13, 28.16, 25.20. **5b** (polar): 7.42-7.25 (m, 5H), 3.97 (t, *J*=8.1 Hz, 1H), 2.48 (m, 1H), 2.45-2.38 (m, 1H), 2.35-2.20 (m, 2H), 2.18-2.04 (m, 2H), 1.93-1.50 (m, 4H), 1.48-1.36 (m, 1H); 211.66, 135.43, 129.12, 128.16, 127.37, 120.88, 47.24, 42.12, 35.54, 34.29, 33.91, 27.79, 25.01.

Preparation of 3-[(2-Methoxymethylene)cyclohexyl]-2-phenylpropanenitrile (6). (Methoxymethyl) triphenylphosphonium chloride (6.02 g, 17.5 mmol) in a 250 mL round-bottomed flask was shortly flame-dried under vacuum and was treated with dry THF (30 mL) and then a 1.5 M phenyllithium solution (12 mL, 18.0 mmol) in cyclohexane/ether at 0 °C under argon atmosphere. The resulting red solution was stirred for 2 h at room temperature, cooled to −78 °C, and treated with ketone 4 (3.05 g, 13.4 mmol) in dry THF *via* cannula. The resulting reaction mixture was warmed to room temperature over 5h and stirred for additional 10 h. The reaction mixture was then cooled to 0 °C and quenched with water (50 mL) and ether (50 mL). The organic products was extracted twice with ether (50 mL × 2), combined, and washed with saturated NaCl solution (100 mL). The organic layer was dried over anhydrous magnesium sulfate, filtered, and concentrated under reduced pressure to give the crude products which mainly contained an isomeric mixture of the desired products. Silica gel chromatography of the crude products gave the corresponding product **6** (1.2156 g, 36%) as a mixture of three diastereomers and a *E*-isomeric product (0.6502 g, 19%).

Product 6: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.42-7.26 (m, 5H), [6.01, 5.97, 5.90 (s, 1H)], 3.78-3.64 (m, 1H), [3.61, 3.58, 3.56 (s, 3H)], [3.22, 2.90, 2.60 (m, 1H)], 2.45-1.20 (m, 10H); ^{13}C NMR (100 MHz, $CDCl_3$) δ For some signals, (141.61, 141.11, 140.97), (137.21, 136.64, 136.51), (129.00, 128.98, 128.88), (127.79, 127.75, 127.68), (127.61, 127.50, 127.04), (121.82, 121.28, 120.83), (117.20, 116.95, 116.74).

Preparation of 4-[(2-Methoxymethylene)cyclohexyl]-3-phenyl-2-butanone (7). The nitrile **6** (1.2156 g, 4.8 mmol) was dissolved in dry ether (10 mL) and treated with a 1.2 M methyllithium solution (10.0 mL, 12 mmol) in ether at −78 °C under argon atmosphere. The resulting mixture was slowly warmed to room temperature and stirred for 30 min, cooled to −78 °C, and quenched with water (10 mL). The organic products was extracted twice with ether (30 mL × 2), combined, and washed with saturated NaCl solution (30 mL). The organic layer was dried over anhydrous magnesium sulfate, filtered, and concentrated under reduced pressure to give the crude products. Silica gel chromatography of the crude products gave the corresponding products (492 mg, 38%) as a mixture of two diastereomers and a mixture of isomeric products (308 mg, 24%). **7a** (nonpolar): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.32-7.18 (m, 5H), 5.86 (d, *J*=2.0 Hz, 1H), 3.66 (dd, *J*=10.8, 3.2 Hz, 1H), 3.50 (s, 3H), 2.82 (m, 1H), 2.20 (ddd, *J*=12.8, 11.2, 4.4 Hz, 1H), 2.06 (s, 3H), 1.97-1.88 (m, 1H), 1.82 (m, 1H), 1.75-1.61 (m, 3H), 1.53-1.40 (m, 3H), 1.24-1.12 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 208.49, 140.50, 140.17, 128.73, 128.11, 126.91, 119.06, 59.01, 56.86, 34.41, 31.86, 31.08, 29.57, 28.25, 26.32, 21.46; FT-IR ($CHCl_3$, cm^{-1}) 1711, 1677, 1598; EIHRMS calcd for $C_{18}H_{24}O_2$ (M^+) 272.1776, found 272.1774. **7b** (polar): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.33-7.18 (m, 5H), 5.79 (d, *J*=1.6 Hz, 1H), 3.62 (t, *J*=6.8 Hz, 1H), 3.54 (s, 3H), 2.73 (m, 1H), 2.58 (ddd, *J*=13.6, 11.2, 6.0 Hz, 1H), 2.07-1.98 (m, 1H), 2.03 (s, 3H), 1.81-1.70 (m, 1H), 1.61-1.37 (m, 6H), 1.24-1.12 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 208.58, 140.34, 139.84, 128.53 (2C), 126.91, 119.22, 59.07, 58.13, 34.38, 32.48, 31.65, 29.01, 28.44, 26.57, 21.68; FT-IR ($CHCl_3$, cm^{-1}) 1706, 1677, 1599; EIHRMS calcd for $C_{18}H_{24}O_2$ (M^+) 272.1776, found 272.1777.

Preparation of Trioxane 3 and 4. An oven-dried 125 mL 3-necked round-bottomed flask was charged with keto enol ether (332.7 mg, 1.10 mmol), methylene blue (*ca*

10 mg) and dry dichloromethane (70 mL). Dry oxygen was bubbled into the solution under UV light generated from medium pressure Hg lamp for 2 h at −78 °C and additional 2h at the range of −78 °C to −35 °C. Oxygen bubbling and the UV light were then turned off and the resulting solution was cooled to −78 °C again and slowly treated with a pre-cooled solution of *tert*-butyldimethylsilyl trifluoromethanesulfonate (0.30 mL, 1.3 mmol) in dichloromethane (1.0 mL) *via* cannula. The reaction mixture was stirred for 10 h at −78 °C and treated with triethylamine (0.7 mL, 5 mmol) and slowly warmed to room temperature. The solution was then transferred to a 250 mL one-necked flask and concentrated under reduced pressure. The residue was separated on silica gel chromatography using a mixture of ethyl acetate and hexane (2:98) as an eluent to give a mixture of impure products (271.5 mg, 50% pure, 41%). This product was dissolved in a 1:10 mixture of ethyl acetate and hexane (1.5 mL) and purified further with HPLC.

3 (nonpolar): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.31-7.18 (m, 5H), 5.03 (d, *J*=1.2 Hz, 1H), 3.59 (s, 3H), 3.47 (dd, *J*=12.6, 3.4 Hz, 1H), 2.28 (ddd, *J*=13.6, 12.8, 12.0 Hz, 1H), 1.89 (bd, *J*=13.6 Hz, 1H), 1.84-1.56 (m, 7H), 1.33-1.14 (m, 2H), 0.99 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 144.21, 128.23(2C), 126.48, 107.22, 104.52, 82.74, 57.07, 53.66, 46.56, 36.82, 35.38, 30.81, 26.07, 24.96, 23.74; FT-IR ($CHCl_3$, cm^{-1}) 1602; CIHRMS calced for $C_{18}H_{25}O_4$ (M+1) 305.1753, found 305.1758. 4 (polar): mp 116-117 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.60-7.57 (m, 1H), 7.34-7.23 (m, 4H), 4.99 (d, *J*=1.2 Hz, 1H), 3.57 (s, 3H), 3.42 (dd, *J*=7.4, 0.8 Hz, 1H), 2.36 (ddd, *J*=14.4, 8.0, 7.2 Hz, 1H), 2.15-2.06 (m, 1H), 1.91 (bd, *J*=14.4 Hz, 1H), 1.74-1.48 (m, 6H), 1.40-1.31 (m, 1H), 1.34 (s, 3H), 1.23-1.16 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 141.30, 130.17, 128.18, 126.66, 105.56, 103.91, 82.70, 57.03, 55.62, 45.25, 35.54, 34.16, 31.40, 25.84, 24.97, 23.77; FT-IR ($CHCl_3$, cm^{-1}) 1602; CIHRMS calced for $C_{18}H_{25}O_4$ (M+1) 305.1753, found 305.1755.

Iron(II) induced Reaction of 4β-Phenyl substituted Trioxane 3. To iron(II) bromide (3.3 mg, 15 mmol) in dry THF (0.5 mL) was added 4-phenyltrioxane **3** (6.5 mg, 21 mmol) in dry THF (0.5 mL) at 0 °C under argon atmosphere. The resulting orange solution was stirred for 1h at room temperature. The solution was cooled to 0 °C and diluted with water (10 mL) and ether (10 mL). The organic layer was extracted twice with ether (10 mL×3), combined, washed with saturated NaCl solution (20 mL), dried over anhydrous magnesium sulfate, filtered, and concentrated under reduced pressure to give the crude products. Silica gel chromatography of the crude products gave an uncharacterizable aldehyde **3a** (0.4 mg, *ca* 6%) and the ring contracted product **3b** (2.8 mg, 43%) as an isomeric mixture. **3a**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.84 (d, *J*=1.6 Hz, 1H), 7.52-7.48 (m, 2H), 7.38-7.26 (m, 3H), 2.52 (dd, *J*=14.0, 12.8 Hz, 1H), 2.42-2.37 (m, 1H), 2.18 (dd, *J*=12.8, 6.0 Hz, 1H), 2.15 (s, 3H), 1.94-1.50 (m, 6H), 1.38-1.26 (m, 2H). **3b**: ^{1}H NMR (400 MHz, $CDCl_3$) δ For major isomer, 7.49 (bd, *J*=7.6 Hz, 2H), 7.34-7.18 (m, 3H), 6.12 (s, 1H), 5.09 (dd, *J*=9.6, 1.6 Hz, 1H), 3.31 (s, 3H), 2.30-2.21 (m, 1H), 2.17 (s, 3H), 2.15-2.02 (m, 2H), 1.93-1.36 (m, 8H), For minor isomer, 6.20 (s, 1H), 5.37 (dd, *J*=9.6, 2.0 Hz, 1H), 3.48 (s, 3H), 2.67-2.58 (m, 1H), 2.20 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.52(2C), 143.18, 138.08, 128.05, 127.80(2C), 126.63, 126.15, 125.47, 98.82, 98.16, 83.96, 83.12, 81.60, 80.91, 57.10, 56.25, 50.49, 47.13, 38.12, 37.26, 35.35, 35.19, 26.05, 25.99, 25.17, 24.98, 22.55, 22.39, 21.18, 21.12; FT-IR ($CHCl_3$, cm^{-1}) 1733; CIHRMS calcd for $C_{14}H_{17}O$ (M-$C_4H_7O_3$) 201.1279, found 201.1281.

Iron(II) induced Reaction of 4α-Phenyl substituted Trioxane 4. The same procedure for trioxane **3** was used. **4a**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.72 (d, *J*=2.0 Hz, 1H), 7.37-7.23 (m, 5H), 5.19 (dd, *J*=9.6, 2.0 Hz, 1H), 2.38-2.27 (m, 2H), 1.96-1.61 (m, 5H), 1.52-1.28 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 202.45, 143.86, 128.32, 127.23, 125.55, 86.62, 79.00, 45.22, 37.65, 31.64, 25.74, 24.84, 21.97; FT-IR ($CHCl_3$, cm^{-1}) 1731, 1604; CIHRMS calcd for $C_{15}H_{19}O_2$ (M+H) 231.1385, found 231.1383. **4b**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.35-7.17 (m, 5H), 5.31 (dd, *J*=9.4, 1.4 Hz, 1H), 4.61 (s, 1H), 3.53 (s, 6H), 2.54 (m, 1H), 2.41 (m, 1H), 1.90-1.65 (m, 5h), 1.58-1.32 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 146.03, 128.01, 126.51, 125.50, 105.01, 84.99, 80.06, 56.96, 54.74, 47.13, 38.26, 35.25, 26.12, 25.08, 22.94; FT-IR ($CHCl_3$, cm^{-1}) 1605; CIHRMS calcd for $C_{17}H_{28}NO_3$ (M+NH_4) 294.2071, found 294.2071. **4c**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36-.7.20 (m, 5H), 3.83 (dd, *J*=8.8, 6.0 Hz, 1H), 2.39-2.32 (m, 7H), 2.28-1.78 (m, 7H), 2.03 (s, 3H), 1.70-1.58 (m, 2H), 1.37 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 213.17, 208.43, 139.08, 128.99, 128.11, 127.29, 56.94, 48.48, 42.16, 34.47, 32.37, 29.32, 27.99, 24.87; FT-IR ($CHCl_3$, cm^{-1}) 1709.

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Purification and Characterization of the Recombinant *Arabidopsis thaliana* Acetolactate Synthase

Kyubong Jo, Seongtaek Hong, Myung-Un Choi, Soo-Ik Chang†, Jung-Do Choi†, and Eun-Hie Koh*

Department of Chemistry, Seoul National University, Seoul 151-742, Korea
†Department of Biochemistry, Chungbuk National University, Cheongju 361-763, Korea
**Department of Chemistry, Duksung Women's University, Seoul 132-714, Korea*
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Acetolactate synthase was purified from *Escherichia coli* MF2000/pTATX containing *Arabidopsis thaliana* acetolactate synthase gene. Purification steps included DEAE cellulose ion exchange column chromatography, phenyl sepharose hydrophobic column chromatography, hydroxylapatite affinity column chromatography, and Mono-Q HPLC. Molecular weight was estimated to be ~65 KDa and purification fold was 109 times. The enzyme showed a pH optimum of 7 and the K_M value was 5.9 mM. The purified enzyme was not inhibited by any of the end products, valine, leucine, and isoleucine.

Introduction

Acetolactate synthase (ALS) is the first common enzyme in the biosynthetic pathways leading to the branched chain amino acids leucine, isoleucine, and valine. It condenses an acetaldehyde moiety derived from pyruvate either with another molecule of pyruvate to form 2-acetolactate or with 2-ketobutyrate to form 2-aceto-2-hydroxybutyrate. ALS has been conserved across species boundaries and substantial sequence homology are observed between the enzymes of bacteria, yeast, and higher plants.[1] Animals do not have the branched-chain amino acid pathways, and therefore, must ingest these amino acids in the diet. In *Escherichia coli* and *Salmonella typhimurium*, three ALS isozymes have been characterized.[2] In these enterobacteria, ALS occurs as a tetramer of two large and two small subunits. The genes encoding each isozyme from *E. coli* had been cloned and sequenced.[3~5] Genes coding for proteins homologous to the large subunit of bacterial ALS have been cloned and sequenced from yeast *Saccharomyces cerevisiae*[6,7] and from the higher plants *Arabidopsis thaliana* and *Nicotiana tabacum*.[1] No small subunit has been demonstrated to be necessary for catalytic activity of either the yeast or the plant ALS enzymes. In addition, regulation of the biosynthesis of valine, leucine and isoleucine in plants is still not fully understood.[8]

ALS is the target of six classes of structurally unrelated herbicides, sulfonylureas,[6,9] imidazolinones,[10] triazolopyrimidines,[11] N-phthalylvaline anilide,[12] sulfonylcarboxamide,[13] and pyrimidyl-oxy-benzoate.[14] ALS enzymes from a wide range of organisms are sensitive to these compounds. Mutants resistant to the herbicides have been described in *Lolium rigidum*,[15~17] *Chlorella emersonii*,[18~20] and *Xanthium strumarium*,[21~23] and resistance has been shown to be attributable to an altered ALS enzyme.

The small crucifer *Arabidopsis thaliana* is commonly