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A stable analog of isochorismate for the study of MenD and other isochorismate-utilizing enzymes

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ABSTRACT

A novel analog of isochorismate, in which the enolpyruvyl substituent is replaced with a carboxymethoxyl group, has been synthesized in four steps from a known intermediate. This analog is more stable than the natural product, but still acts as a good substrate for the enzyme MenD (SEPHCHC synthase). The enzyme consumes the (+)-enantiomer only, with an apparent turnover similar to that of the natural substrate, and an apparent Michaelis constant conveniently higher than that of isochorismate. This analog will be useful in the study of any isochorismate-utilizing enzyme.

(+)-Isochorismate, shown in Figure 1, is a metabolite found in bacteria and plants that is used in the biosynthesis of salicylic acid,[1] enterobactin,[2] and menaquinone (vitamin K).[2,3] Isochorismate is synthesized from (−)-chorismate in a reversible reaction catalyzed by isochorismate synthase (EC 5.4.4.2), which is found as an enterobactin-specific enzyme EntC,[4] and a menaquinone-specific enzyme MenF.[5] Other enzymes utilizing isochorismate include isochorismatase, also called isochorismate-pyruvate hydrolase or EntB, EC 3.3.2.1;[6] isochorismate-pyruvate lyase, PchB;[7] and 2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate (SEPHCHC) synthase, MenD (EC 2.2.1.9).[8] Isochorismate is also known to undergo spontaneous reactions, including Claisen rearrangement to isoprephenate and elimination to salicylate and pyruvate, as described by Hilvert.[9]

Our laboratory has been studying MenD from *Escherichia coli*.[10,11] This enzyme catalyzes a thiamine diphosphate (ThDP)- and Mg^{2+}-dependent reaction of isochorismate with α-ketoglutarate to form SEPHCHC and CO_2 (Scheme 1), the first unique step in the biosynthesis of menaquinone. Many bacteria, including *Mycobacterium tuberculosis*, rely on menaquinone as a terminal electron acceptor. Controlling menaquinone levels in such bacteria has been shown to affect their ability to persist in hypoxic environments,[12] and therefore the menaquinone pathway has been proposed as a target for antibiotic development.[13] MenD has also attracted interest as a novel catalyst for asymmetric synthesis.[14]

The tendency of isochorismate to decompose at room temperature, as well as its very low apparent K_m in the MenD reaction, led

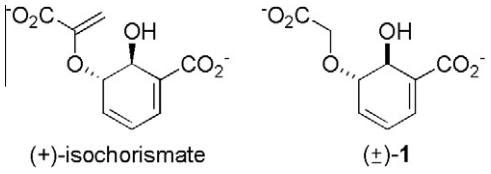
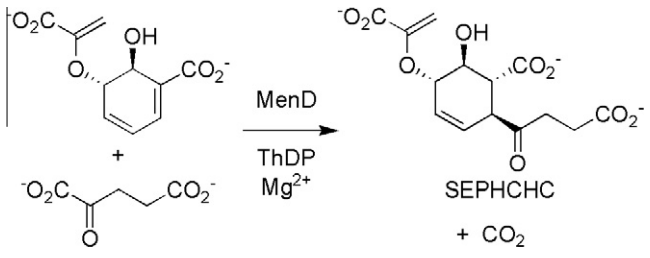


Figure 1. Structures of naturally occurring isochorismate and the isochorismate analog **1**.



Scheme 1. The reaction catalyzed by MenD: ThDP-dependent decarboxylation of α-ketoglutarate and carboligation to form SEPHCHC.

Abbreviations: EntB, isochorismatase; EntC, enterobactin-specific isochorismate synthase; MenD, SEPHCHC synthase; MenF, menaquinone-specific isochorismate synthase; PchB, isochorismate-pyruvate lyase; SEPHCHC, 2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate; SHCHC, 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate; ThDP, thiamine diphosphate.

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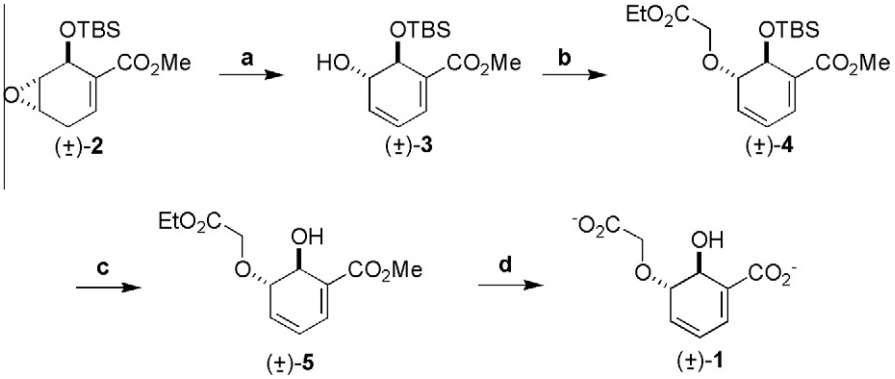


Scheme 2. Synthesis of (±)-**1**. Reagents: (a) DBU (5.0 equiv), CH_2Cl_2, 85%. (b) $N_2CHCOEt$, $Rh_2(OAc)_4$, CH_2Cl_2, 71%. (c) AcOH/H_2O (v/v 3:2), 84%. (d) LiOH, THF/H_2O (v/v 4:1).

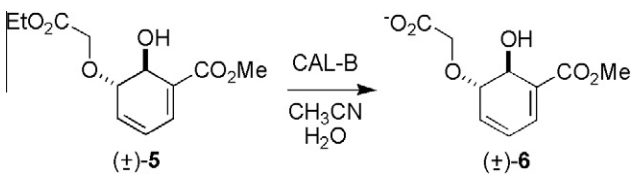
us to seek an isochorismate analog that would be more robust than the natural product, while remaining a good substrate for the enzyme. This would allow more extensive investigation of the reaction and mechanism of the wild-type MenD and other enzymes. The structural perturbation to the substrate can also demonstrate the importance of structural features of the substrate in the enzyme–substrate interaction. Removal of the carbon–carbon double bond from the enolpyruvyl substituent would result in a poorer leaving group, and therefore a structure more resistant to spontaneous salicylate formation. Such a compound would also be unable to undergo the pericyclic reaction mechanism proposed for the PchB reaction,[7,15] making it a likely inhibitor for such reactions, useful for crystallographic and mechanistic studies. Here we describe the synthesis of *trans*-5-carboxymethoxy-6-hydroxy-1,3-cyclohexadiene-1-carboxylate (**1**) as a racemic mixture. We show that it is a useful substrate for the study of MenD, and may also be of use in the understanding of MenF, EntC, EntB, PchB, and related enzymes.

*Synthesis of analog **1***. Synthesis of **1** was carried out as shown in Scheme 2. Oxirane **2** was synthesized using the method of Bartlett;[16] briefly, Diels–Alder addition of the *tert*-butyldimethylsilyl enol ether of crotonaldehyde to the dienophile methyl propiolate generated a 1,4-cyclohexadienyl ring, which underwent chemo- and stereoselective epoxidation using *m*-CPBA to give the racemic *trans*-product **2** exclusively.

Epoxide ring opening was effected using 5 equiv of DBU in dry DCM under argon atmosphere for 16 h at room temperature, followed by silica gel FCC in 3:1 hexane/ethyl acetate, providing alcohol **3** in 85% yield as a colorless oil. This alcohol was alkylated using ethyl diazoacetate in dry DCM in the presence of 1 mol % $Rh_2(OAc)_4$, followed by FCC using 10–50% ethyl acetate in hexane, resulting in recovery of unreacted starting material, and the alcohol **4** in 71% yield based on consumed starting material. The TBS ether was removed in 84% yield by stirring in 3:2 acetic acid/water at room temperature for 16 h, followed by FCC in 3:2 hexane/ethyl acetate, resulting in diester **5** as a colorless liquid. The ester groups were hydrolyzed using 6 equiv of LiOH in 4:1 THF/H_2O at room temperature, and the product purified by ion exchange chromatography to give the target **1** as either the dilithium salt or as the free diacid, depending on form of resin used, in 45–55% yield. Characterization of this novel compound included polarimetric confirmation that the product was racemic.

Enhanced stability of **1** relative to isochorismate was evident in handling of the compound. We observed no decomposition of **1** when stored at 4 °C for weeks, and NMR spectra were unchanged. This is in contrast to isochorismate, which is not stable under such conditions; concentrations of even freshly prepared solutions of isochorismate must be determined enzymatically. As pointed out by Hilvert, Claisen rearrangement is the primary route of decomposition;[9] such rearrangement is not possible for **1**.

The racemic product was found to be convenient for enzymatic assays, since the (−)-enantiomer was neither substrate nor inhibitor of the enzyme, having no apparent effect on the reaction (see below). However, we did investigate whether an enantioselective hydrolysis of the esters could be effected using a commercially available lipase from *Candida antarctica* (CAL-B). Although we could not observe enantioselective hydrolysis, stirring for 2.5 h at room temperature in 1:1 acetonitrile/H_2O gave chemoselective cleavage of the ethyl ester from the carboxymethoxyl moiety, as shown in Scheme 3, to obtain **6** in quantitative yield. This compound was not a substrate for MenD. Characterization of **1** included UV–vis spectroscopy, which indicated that this analog had an absorbance maximum near 278 nm, in simile with isochorismate. The extinction coefficient was determined to be



Scheme 3. Selective hydrolysis of the ethyl ester of **1** using *Candida antarctica* lipase B (CAL-B).

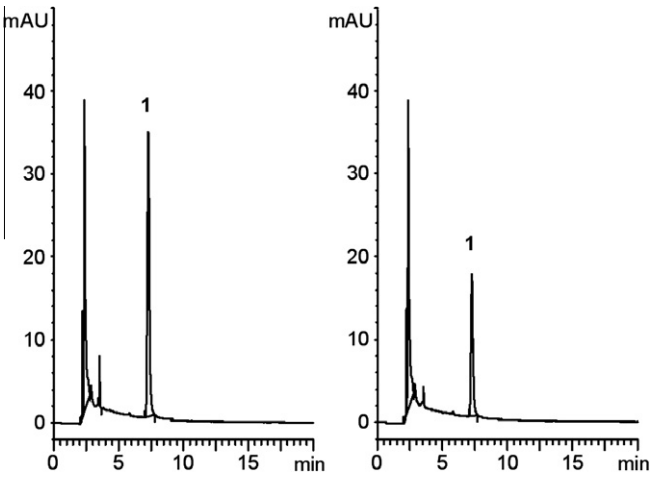


Figure 2. HPLC chromatogram, detection at 278 nm, showing the peak due to **1** at the beginning, $t = 0$ (left) and after, $t = 30$ min (right) the reaction with α-ketoglutarate in the presence of MenD, ThDP, and $MgCl_2$. Zorbax SB-C18 (4.6 × 250 mm) analytical column, 5% acetonitrile and 95% aqueous formic acid (0.1%), flow rate = 1 mL/min. Calculated area of the peak due to **1** = 379.5 (left) and 188.6 (right).

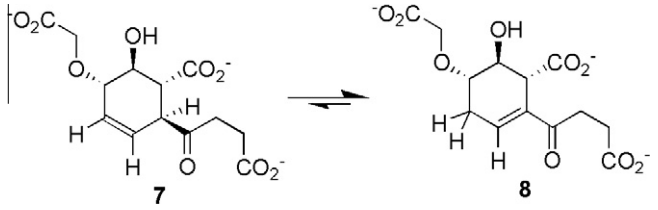
4700 M^{-1} cm^{-1}. All compounds were characterized by NMR spectroscopy.[17]

MenD-catalyzed reaction of ***1***. MenD and (+)-isochorismate were prepared and purified as described previously.[11] Reactions were carried out in 100 mM Tris buffer at pH 7.4, using 100 μM α-ketoglutarate, 50 μM ThDP, and 5 mM $MgCl_2$. The consumption of **1** in the presence of MenD, α-ketoglutarate, ThDP, and Mg^{2+} was evaluated by reverse-phase HPLC, as shown in Figure 2. Integration of the peak due to **1** before and after incubation with enzyme for 30 min showed that 50% of the peak was consumed, whereas in the analogous experiment using (+)-isochorismate, the peak due to substrate was completely consumed (data not shown). The remaining peak due to unconsumed **1** was isolated by semi-preparative reverse-phase HPLC and characterized by ^1H NMR and polarimetry. The NMR spectrum was identical to that of racemic **1**, but the specific rotation due to this compound was −170 (*c* 0.24 H_2O), indicating that (+)-**1** had been consumed in the reaction. For comparison, (+)-isochorismate synthesized enzymatically in our laboratory had a specific rotation of +219 (*c* 0.18 H_2O). The isolated (−)-**1** was tested as a substrate for MenD, resulting in no apparent activity. Furthermore, this enantiomer did not have any effect on the MenD-catalyzed reaction of (+)-isochorismate; being neither substrate nor inhibitor strongly suggests that (−)-**1** does not bind to the active site of MenD.

Extended incubation of the MenD—**1** reaction mixture resulted in the appearance of a peak in the HPLC chromatogram with a considerably longer retention time, and an absorbance maximum at 228 nm. Isolation and analysis of this compound revealed it to be the α,β-unsaturated compound **8** shown in Scheme 4. Spontaneous isomerization of the enzymatic product **7** to the more stable, conjugated **8** is not surprising, although a similar product resulting from the isochorismate-derived product SEPHCHC has not been observed because SEPHCHC undergoes spontaneous elimination to form 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate (SHCHC).

The MenD-catalyzed reaction of **1** with α-ketoglutarate can be measured cleanly at 278 nm, and the drop in absorbance was consistent with consumption of only one enantiomer. The analogous reaction of (+)-isochorismate is followed by a slow increase in absorbance due to the formation of SHCHC, which absorbs strongly at 293 nm. When **1** is used as substrate, this increase is absent, demonstrating the enhanced stability of product **7**. When the isolated enzymatic reaction product **7** was incubated at pH 13, SHCHC formed (data not shown).

Figure 3 shows the dependence of initial rate of the MenD-catalyzed reaction of **1** on substrate concentration in the presence of saturating α-ketoglutarate concentration (100 μM). The apparent K_m value is 24 ± 2 μM, but given the evidence that only the (+)-enantiomer is consumed, the apparent K_m value of (+)-**1** is 12 μM. This is at least an order of magnitude higher than the K_m of the natural substrate, but is in the range of many naturally occurring enzymatic substrates. Surprisingly, the apparent k_{cat} for (+)-**1** is 24 ± 2 s^{-1}, slightly higher than the value of 17 ± 3 s^{-1} measured in our laboratory using (+)-isochorismate as the substrate.[11]



Scheme 4. Non-enzymatic isomerization of **7**, the enzymatic product derived from (+)-**1**, to **8**.

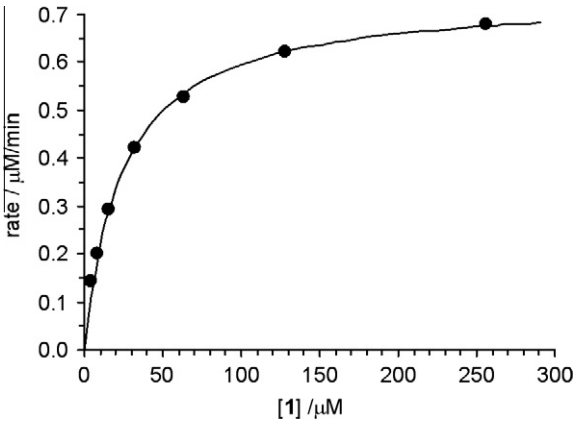


Figure 3. Dependence of rate of substrate consumption on concentration, measured at 278 nm. Each point (●) represents the average of at least two experiments, and the line indicates the fit to the Michaelis–Menten equation, as determined using the program Leonora.[18]

In conclusion, **1** can be synthesized in good yield and used to take the place of isochorismate in an enzymatic reaction. This compound is more stable than isochorismate, and can therefore be helpful in the functional and/or structural study of several isochorismate-utilizing enzymes.

Acknowledgments

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References and notes

1. Strawn, M. A.; Marr, S. K.; Inoue, K.; Inada, N.; Zubieta, C.; Wildermuth, M. C. *J. Biol. Chem.* **2007**, *282*, 5919.
2. Buss, K.; Muller, R.; Dahm, C.; Gaitatzis, N.; Skrzypczak-Pietraszek, E.; Lohmann, S.; Gassen, M.; Leistner, E. *Biochim. Biophys. Acta-Gene Struct. Expr.* **2001**, *1522*, 151.
3. Meganathan, R. *Vitam. Horm.* **2001**, *61*, 173.
4. Sridharan, S.; Howard, N.; Kerbarh, O.; Blaszczyk, M.; Abell, C.; Blundell, T. L. *J. Mol. Biol.* **2010**, *397*, 290.
5. Kolappan, S.; Zwahlen, J.; Zhou, R.; Truglio, J. J.; Tonge, P. J.; Kisker, C. *Biochemistry* **2007**, *46*, 946.
6. Gehring, A. M.; Bradley, K. A.; Walsh, C. T. *Biochemistry* **1997**, *36*, 8495.
7. Luo, Q. Y.; Olucha, J.; Lamb, A. L. *Biochemistry* **2009**, *48*, 5239.
8. Jiang, M.; Cao, Y.; Guo, Z. F.; Chen, M. J.; Chen, X. L.; Guo, Z. H. *Biochemistry* **2007**, *46*, 10979.
9. DeClue, M. S.; Baldridge, K. K.; Kast, P.; Hilvert, D. *J. Am. Chem. Soc.* **2006**, *128*, 2043.
10. Bhasin, M.; Billinsky, J. L.; Palmer, D. R. J. *Biochemistry* **2003**, *42*, 13496.
11. Fang, M.; Toogood, R. D.; Macova, A.; Ho, K.; Franzblau, S. G.; McNeil, M. R.; Sanders, D. A. R.; Palmer, D. R. J. *Biochemistry* **2010**, *49*, 2672.
12. Dhiman, R. K.; Mahapatra, S.; Slayden, R. A.; Boyne, M. E.; Lenaerts, A.; Hinshaw, J. C.; Angala, S. K.; Chatterjee, D.; Biswas, K.; Narayanasamy, P.; Kurosu, M.; Crick, D. C. *Mol. Microbiol.* **2009**, *72*, 85.
13. Lu, X. Q.; Zhang, H. N.; Tonge, P. J.; Tan, D. S. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 5963.
14. Kurutsch, A.; Richter, M.; Brecht, V.; Sprenger, G. A.; Muller, M. *J. Mol. Catal. B: Enzym.* **2009**, *61*, 56.
15. DeClue, M. S.; Baldridge, K. K.; Kunzler, D. E.; Kast, P.; Hilvert, D. *J. Am. Chem. Soc.* **2005**, *127*, 15002.
16. Kozlowski, M. C.; Tom, N. J.; Seto, C. T.; Sefler, A. M.; Bartlett, P. A. *J. Am. Chem. Soc.* **1995**, *117*, 2128.
17. All NMR spectra were acquired using a Bruker 500 MHz spectrometer with samples dissolved in the appropriate deuterated solvents ($CDCl_3$, CD_3OD, and D_2O). Chemical shift is reported in ppm downfield from tetramethylsilane

based on solvent residual peaks. Specific rotations ($[\alpha]_D$) were determined at ambient temperature using a 1-mL, 10-dm cell; the units are 10^{-1} deg cm^2 g^{-1}, the concentrations (*c*) are reported in g/100 mL, and the values are rounded to reflect the accuracy of the measured concentrations (the major source of error). Mass spectrometry was performed on mass spectrometer API Qstar XL pulsar hybrid LC/MS/MS system with Agilent 1100 series HPLC or an integrated syringe pump. Melting points were measured on a Gallencamp melting point apparatus and are not corrected. Compound **3**: R_f = 0.17 in 25% EtOAc/hexane. ^{1}H NMR ($CDCl_3$): δ 7.11 (d, 1H, *J* = 5.5 Hz), 6.32 (dd, 1H, *J* = 9.0, 5.5 Hz), 6.26 (dd, 1H, *J* = 9.0, 5.5 Hz), 4.68 (s, 1H), 4.13 (m, 1H), 1.44 (d, 1H, *J* = 7.5 Hz, OH), 0.82 (s, 9H), 0.2 (s, 3H), 0.1 (s, 3H). ^{13}C NMR ($CDCl_3$): δ 167.1, 132.5, 132.5, 129.6, 124.5, 69.3, 67.7, 51.7, 25.7, 18.1, −4.5, −4.8. HRMS (CI^+): calcd for $C_{14}H_{25}O_4Si$ 285.1522, found 285.1528 ($[M+1]^+$). Compound **4**: R_f = 0.32 in 25% EtOAc/hexane. ^{1}H NMR ($CDCl_3$): δ 7.10 (d, 1H, *J* = 5.5 Hz), 6.35 (dd, 1H, *J* = 9.5, 5.5 Hz), 6.26 (dd, 1H, *J* = 9.5, 5.0 Hz), 4.75 (s, 1H), 4.20 (q, 2H, *J* = 7.0 Hz), 4.06 (m, 3H, *CHOCH*$_2$), 3.76 (s, 3H), 1.27 (t, 3H, *J* = 7.0 Hz), 0.80 (s, 9H), 0.16 (s, 3H), 0.06(s, 3H). ^{13}C NMR ($CDCl_3$): δ 170.4, 170.0, 132.8, 130.1, 129.3, 126.3, 76.4, 65.4, 65.1, 61.1, 51.8, 25.0, 18.1, 14.3, −4.4, −4.7. HRMS (EI^+): calcd for $C_{18}H_{30}O_6Si$ 370.1812, found 370.1820 ($[M]^+$). Compound **5**: R_f = 0.18 in 40% EtOAc/Hexane. ^{1}H NMR ($CDCl_3$): δ 7.01 (d, 1H, *J* = 5.5 Hz), 6.30 (dd, 1H, *J* = 9.0, 3.0 Hz), 6.16 (ddd, 1H, *J* = 9.5, 5.5, 1.5 Hz), 4.88 (d, 1H, *J* = 6.5 Hz), 4.34 (ddd, 1H, *J* = 6.5, 3.0, 1.5 Hz), 4.28 (s, 2H), 4.21 (q, 2H, *J* = 7.0 Hz), 3.80 (s, 3H), 3.43 (s, 1H), 1.28 (t, 3H, *J* = 7.0 Hz). ^{13}C NMR ($CDCl_3$): δ 170.7, 167.5, 133.0, 132.8, 129.6, 123.9, 79.6, 69.5, 67.0, 61.1, 52.2, 14.3. HRMS (EI^+): calcd for $C_{12}H_{16}O_6$ 256.0947, found 256.0942 ($[M]^+$). Compound **1**: ^{1}H NMR (D_2O): δ 6.82 (d, 1H, *J* = 5.5 Hz), 6.32 (dd, 1H, *J* = 10.5, 5.5 Hz), 6.19 (dd, 1H, *J* = 9.5, 5.0 Hz), 4.70 (d, 1H, *J* = 3.0 Hz), 4.11 (m, 1H), 3.95 (s, 2H). ^{13}C NMR (D_2O): δ 177.9, 174.4, 134.0, 130.0, 127,5, 126.5, 76.6, 66.9, 65.9. HRMS (ESI^-): calcd for $C_9H_8LiO_6$ 219.0486, found 219.0490 ($[M-Li]^-$). Compound **6**: ^{1}H NMR (D_2O): δ 7.13 (d, 1H, *J* = 6.0 Hz), 6.34 (dd, 1H, *J* = 9.5, 6.0 Hz), 6.26 (dd, 1H, *J* = 9.5, 5.0 Hz), 4.61 (s, 1H), 4.09 (s, 2H), 4.07 (s, 1H, *J* = 5.0 Hz), 3.68 (s, 3H). Compound **8**: $[\alpha]_D^{25} = -48 \pm 5$ (*c* 0.12, H_2O). ^{1}H NMR (CD_3OD): δ 7.11 (br, 1H), 4.10 (m, 3H), 3.62 (m, 1H), 3.33 (d, 1H, *J* = 6.0 Hz), 3.03 (m, 1H) & 2.93 (m, 1H), 2.75 & 2.71 (m, m, 1H), 2.49 (t, 1H, dd, 1H, *J* = 6.0 Hz), 2.40 & 2.35 (m, m, 1H). ^{13}C NMR ($CDCl_3$): δ 201.3, 177.4, 176.5, 174.7, 142.1, 133.8, 76.5, 70.2, 66.9, 47.5, 31.8, 29.6, 27.9. HRMS (ESI^-): calcd for $C_9H_{15}O_9$ 315.0722, found 315.0741 ($[M-H]^-$).

18. Cornish-Bowden, A. *Analysis of Enzyme Kinetic Data*; Oxford University Press: New York, 1995.