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Conversion of Aldonic Acids to Their Corresponding 2-Keto-3-deoxy-analogs by the Non-Carbohydrate Enzyme Dihydroxy Acid Dehydratase (DHAD)

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Abstract—Aldonic acids containing four to six carbon atoms were tested as potential substrates of dihydroxy acid dehydratase (DHAD), an enzyme from the biosynthetic pathway of branched chain amino acids. Novel assay systems for observing the course of DHAD catalysed reactions were developed in order to adapt the enzyme to extended practical applications. Kinetic studies for the new substrates (**12**/**13**) as well as inhibitor kinetics for the substrate analogue 3-deoxy-aldonic acids (**25**, **27** and **29**) were performed. These gave indications for the restrictions of substrate modifications and contributed to the understanding of the individual effects. Finally L-threonic acid (**12**) and D-erythronic acid (**13**) could be successfully applied as substrates for DHAD and this led to the chemoenzymatic synthesis of their 2-keto-3-deoxy-analogue (**20**) in a preparative scale.

Introduction

DHAD catalyses the dehydration of its two natural substrates **3** and **4** to give the corresponding 2-keto acid analogs **5** and **6**. The enzyme is involved in the biosynthesis of the branched chain amino acids valine (**7**), isoleucine (**8**) and leucine (**9**) in higher plants, bacteria, algae and yeasts.[1] The enzyme has been isolated from both plant and bacterial sources.[2] Among the plant sources spinach leaves yielded extracts with the highest specific activity of DHAD and this was used most frequently as the source of the enzyme.[3] DHAD from all sources shows similar properties such as a pH optimum around 8 and optimal enzyme activity in the presence of divalent metal ions[2] such as Mg^{2+}. Heavy metal ions, fluorides and chelating agents such as EDTA are known as inhibitors of DHAD.[2] The enzyme contains two identical subunits of 65 to 66 kDa.[4]

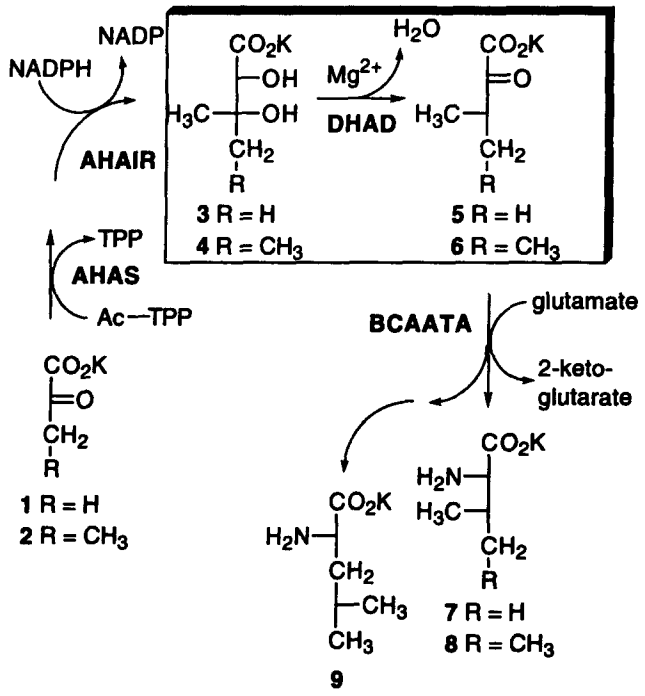
For substrate recognition by DHAD a 2,3-dihydroxy acid with 2(*R*) configuration is essential. In the case of the diastereomeric substrates **4** and **4a** both the natural 2(*R*),3(*R*) isomer **4** and the artificial 2(*R*),3(*S*) isomer **4a** are accepted as substrates for DHAD. The reaction of the artificial *threo*-dihydroxy acid **4a** was reported to be even faster than for the natural *erythro*-isomer.[5]

The catalytic mechanism of DHAD has been partly investigated. By labelling experiments Arfin *et al.* [6] found evidence for an enol intermediate in the course of the reaction. Later results proved a stereoselective rearrangement of the enzyme-bound enol towards the final 2-keto acid. Flint *et al.*[7] observed an iron–sulphur cluster located at the active site of the enzyme and postulated that it was involved in the catalytic process in the initial cleavage of the hydroxy group at C-3 like a Lewis acid. Pirrung *et al.*[8] gave evidence for this postulated cationic-like intermediate and also proved an enzyme-bound enol by studying intermediate analogue inhibitors. The main results of these findings are depicted in Scheme 2 as a postulated catalytic mechanism.

Only a few studies in substrate modifications have been carried out. Armstrong *et al.*[9] tested substrates with longer aliphatic chains at C-3 and observed low activity even for a dihydroxy acid containing a seven-carbon chain. Pirrung *et al.*[8] studied the influence of +I and –I substituents at C-3 and observed that the turnover rates were significantly diminished by introduction of a fluoromethyl or a trifluoromethyl instead of a methyl group. However, substitution of one methyl group by a cyclopropyl group led to no significant change in reaction yields compared to the natural substrate **3**.

Previous investigations employed DHAD in the production of the natural products valine (**7**) and isoleucine (**8**) by fermentation[10] and synthesis of the natural keto acids **5** and **6** on a milligram scale.[8] However, the enzyme has never been used before in a chemoenzymatic synthesis nor have carbohydrates been used as substrates. The present idea was to take advantage of this easily accessible enzyme for a new chemoenzymatic synthesis of 2-keto-3-deoxy-aldonic acids starting with aldonic acids. The desired compounds are involved in many biological processes such as the oxidative degradation of aldopentoses and -hexoses,[11] the 2-keto-3-deoxy-D-arabino-heptonic acid[12] in the shikimic acid pathway that produces aromatic amino acids and other aromatic natural products such as vitamin K. The 2-keto-3-deoxy-aldonic acids containing eight and nine carbon atoms such as 2-keto-

3-deoxy-D-manno-octonic acid (KDO)[13] and sialic acids[14] are components of bioactive molecules and play an important role in many molecular recognition processes.

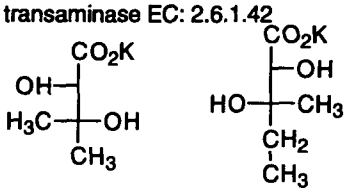


AHAS: Acethydroxy acid synthetase
EC: 4.1.3.18

AHAIR: Acethydroxy acid isomeroreductase
EC: 1.1.1.86

DHAD: Dihydroxy acid dehydratase
EC: 4.2.1.9

BCAATA: Branched chain amino acid transaminase EC: 2.6.1.42



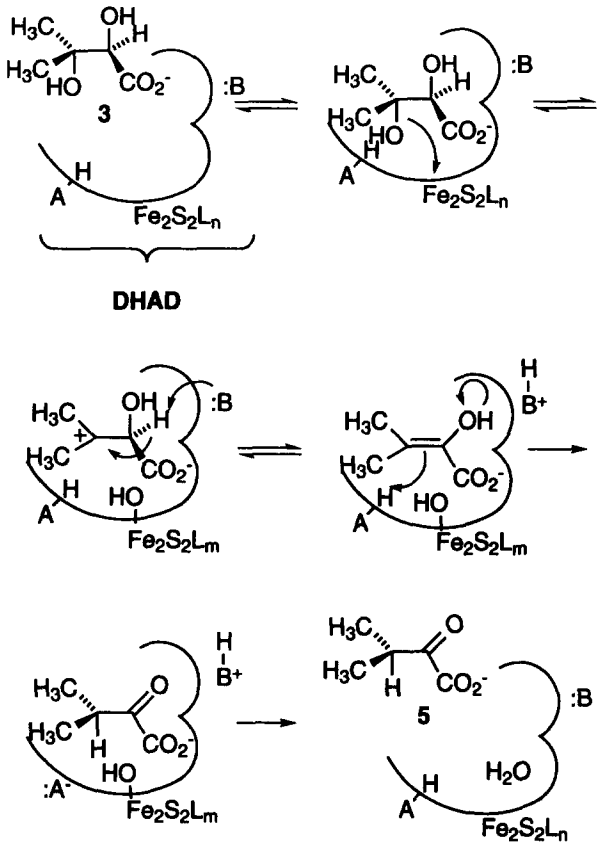
3a **4a**

2(S)-enantiomer of **3** 2(R),3(S)-enantiomer of **4**

Scheme 1.

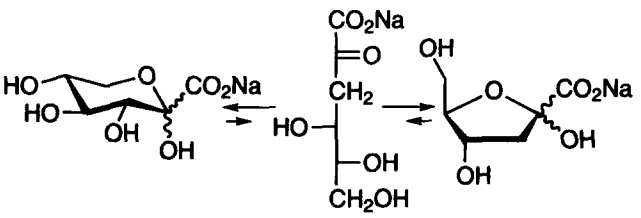
Results and Discussion

These investigations were started with the intention of using DHAD for a chemoenzymatic synthesis on a preparative scale. For that reason spinach leaves were chosen which are easily accessible in unlimited quantities as the source of the enzyme. The purification was carried out as described by Pirrung *et al.* with slight modifications.[15] It started with homogenisation in Tris–HCl buffer, polymin P and successive ammonium sulphate precipitations followed by anion exchange chromatography on DEAE cellulose. This material seemed to be sufficiently pure for the initial purpose,



Scheme 2.

that is the application of the enzyme to a preparative synthesis. However, it had to be demonstrated that there were no activities which would interact with either the starting material or the formed product. This was shown by incubation for seven days at 35 °C of the partially purified protein fraction with the commercially available natural product **5** and chemically synthesised 2-keto-3-deoxy-D-threo-hexonic acid (**10**)[16] as a representative of the class of compounds intended to be synthesised.



10

Scheme 3.

Until now the only analytical tool employed for the observation of the enzymatic conversion was a colorimetric assay with 2,4-dinitrophenylhydrazine.[17] This assay is based on hydrazone formation with the enzymatically formed α-keto acid and uses the photometric measurement of the absorbance in alkaline pH. In addition to the disadvantage of the long processing time of more than 1 h the influence of acetalisation of the keto group, as usually found in

keto-aldonic acids such as **10** was not proved. Thus it was decided to adapt an assay with semicarbazide (SCA-assay), which was originally developed for the quantitative measurement of 2-keto-aldonic acids.[18] The benefit of the new assay is that the absorbance can be measured photometrically at 250 nm directly in the initial acidic medium and the processing time can be shortened to only 20 min. Both the amount of natural product **5** and artificial product **10** could be detected by the SCA-assay and showed linearity in an extended range of concentrations. The disadvantage of this new assay is that the region of the absorbance maximum of the semicarbazone at 250 nm would be seriously interfered with by large quantities of protein. Thus the SCA-assay could not be used for crude enzyme preparations.

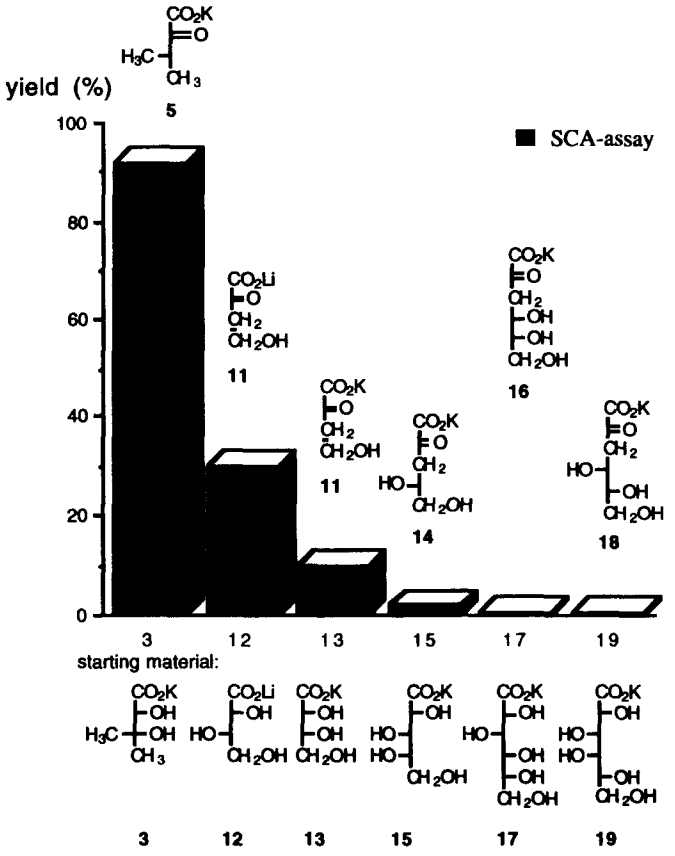
For analytical investigation of the substrate capacity of the artificial substrates **12**, **13**, **15**, **17** and **19** 10 mM solutions of each substrate together with 20 mU of the purified enzyme obtained after anion exchange chromatography were incubated. The amount of product formation was monitored by the SCA-assay which showed 92% conversion for the natural substrate **3** after incubation at 35 °C for three days. The results for the aldonic acids **3**, **12**, **13**, **15**, **17** and **19** are summarised in Scheme 4.

As shown in Scheme 4 the aldotetronic acids **12** and **13** could be used as substrates for DHAD. However, the difference in the yields for conversion of **12** and **13** indicated a strong influence of the configuration at C-3, which is evaluated in detail by measurement of enzyme kinetics for these substrates. The results of these kinetic studies are shown in Table 1.

Table 1.

Substrate (x)	K_m (mM)	$V_{rel} = \frac{V_{max}(3)}{V_{max}(x)}$
natural substrate (**3**)	0.48	1.00
L-threonate (**12**)	0.65	0.31
D-erythronate (**13**)	1.75	0.02

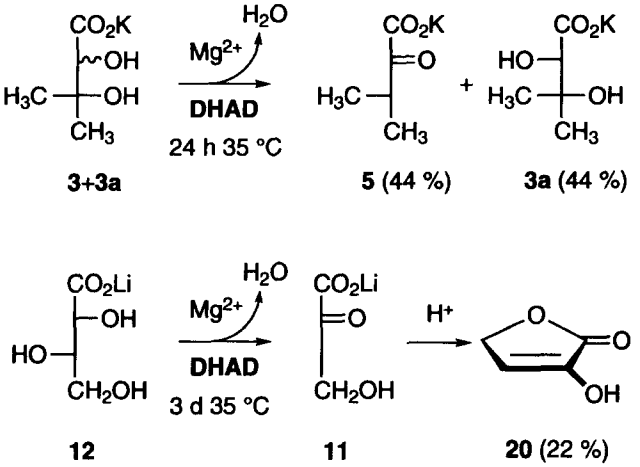
The K_m value of 0.48 mM for the natural substrate **3** corresponded to earlier published data.[7,15,19] For the aldonic acids **12** and **13** the K_m values were a little



Scheme 4.

higher but still in the same range of magnitude. Substitution of the two methyl groups of the natural substrate **3** by only one hydroxymethyl group, such as in the artificial substrates **12** and **13**, caused no noteworthy reduction in binding to the enzyme as observed by comparison of the relative reaction rates. It may be assumed that the missing +I effect of the original methyl groups and the additional –I effect of the unnatural hydroxymethyl group induced a destabilisation of the initially formed cationic-like intermediate in the course of the enzymatic mechanism which led to the observed deceleration of the reaction velocity. The preference for the *threo* over the *erythro* configured dihydroxy acids, previously also observed for the two diastereomers of the natural substrate, **4** and **4a**, seemed to be reinforced for **12** and **13** by the electronic destabilisation effect outlined above. On this basis L-threonate (**12**) was chosen as the starting material for a representative scaling-up of the enzymatic reaction to a preparative size.

As an additional analytical technique an HPLC system based on a HPX 87 column (Aminex) for separations of organic acids was developed. This method allowed the monitoring of product formation and consumption of starting material simultaneously and could be used for preparative separations. In a preparative conversion, 1 mmol racemic **3** + **3a** and 2 U DHAD were incubated for 24 h at 35 °C, and this led to the isolation of 44% product **5** in addition to 44% unreacted starting material **3** + **3a**, highly enriched by the inactive 2(*S*)-enantiomer **3a**.



Scheme 5.

For better enzyme stability and easier removal of the proteins, DHAD was immobilized on activated CH Sepharose 4B in 86% activity yield by standard procedures.[20] The work-up of the incubation mixture containing **3** + **3a** and **5** started with filtration of the immobilised material followed by removal of Tris by treatment with a cationic exchange resin and freeze drying. Finally, separation of starting material, product and inorganic buffer salts was achieved by HPLC.

In the preparative conversion of **12**, initial observations indicated a slow decomposition of product **11** after three days. Several further additions of DHAD led to complete consumption of the starting material but could not increase the amount of **11**. The open chain acid **11** was known to be labile and not to be isolated.[21] However, the corresponding γ-lactone **20** could be isolated. Comparable incubations of chemically synthesised **20**,[16] which was previously converted to **11** by titration with a solution of lithium hydroxide, with and without enzyme showed the same rate of decomposition. For a preparative chemoenzymatic conversion of **12** by DHAD the ratio of enzyme to substrate was changed to 0.1 mM substrate concentration with 2 U enzyme in order to achieve a faster conversion. The work-up procedure was carried out as described above until treatment with cationic exchange resin. The acidic solution obtained was partly concentrated by lyophilisation and the lactonised product was isolated by extraction with diethylether to give **20** in 22% yield.

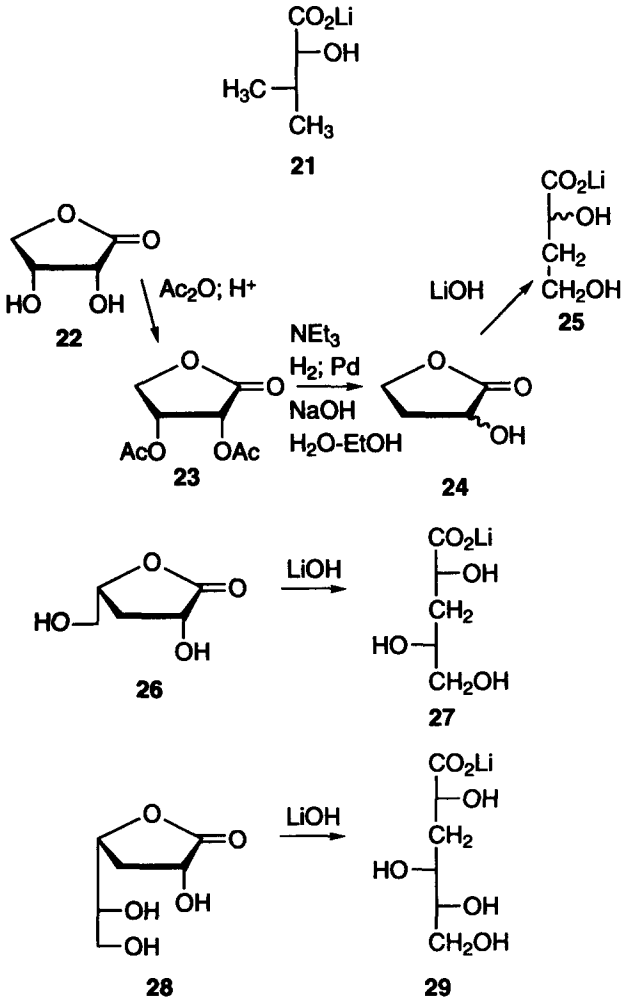
In the case of the other aldonic acids, **15**, **17** and **19** had very poor yields which prohibited a chemo-enzymatic conversion on a preparative scale. In particular, for L-arabinonic acid (**15**) the conversion rate of 2% was suprisingly low. Steric reasons for considerably less perfect binding at the active site of the enzyme cannot be expected, because the natural substrate **4** also contains a five-carbon chain. In order to evaluate reasons for these findings the ability of the 3-deoxy analogues **25**, **27** and **29** of **15**, **17** and **19** to act as inhibitors was studied. This was intended to distinguish between steric reasons for a hindered binding to the enzyme and electronic effects for slow conversion as demonstrated for the aldotetronic acids **12** and **13**. As has been shown for the substrate analogue inhibitor 2-hydroxy isovaleric acid (**21**) [15] the 3-deoxy acids **25**, **27** and **29** should show competitive inhibition in the case of binding at the active site.

The 3-deoxy acids **25**, **27** and **29** were obtained by titration with lithium hydroxide of their corresponding 3-deoxy-lactones **24**, **26** and **28** which in turn could be easily synthesised as described for **26** and **28** by Pedersen *et al.*[22]

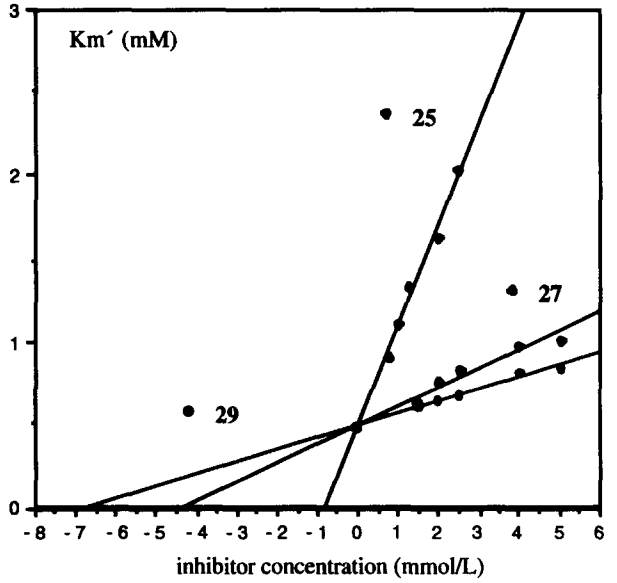
The inhibitor kinetics were measured for concentrations between 0.1 mM and 10 mM for substrate **3** and between 1 mM and 5 mM concentrations of each inhibitor **25**, **27** and **29**. The results are summarised in Scheme 7 and Table 2.

The K_i values for the 3-deoxy acids **25**, **27** and **29** clearly demonstrate that the binding capacity decreased with increasing chain length of the inhibitor. Even for the aldonic acid **29** a remarkable inhibitor potential was observed. This led to the conclusion that the principle reason for the poor reaction of **15**, **17** and **19** was caused by electronic destabilisation effects due to the electron withdrawing hydroxy groups. The hydroxy group in position 4 may play the most important role

because it is situated next to the cationic-like centre at C-3 which is initially formed in the course of the enzymatic mechanism.



Scheme 6.



Scheme 7.

Table 2.

Inhibitor (x)	$K_i(x)/K_m(3)$
21	6.0 (Ref. 15)
25	1.7
27	9.2
29	14.2

In order to evaluate the influence of the electronic nature of the substituents at C-4, investigations with several modified aldopentonic acids were started. Substitution of the hydroxy group at C-4 in **15** by substituents with different electronic withdrawing or donating effects should help to out line the significance of this position for the enzymatic conversion rates. The results of this investigation will be published in due course.

Conclusion

Aldonic acids are able to act as substrates for DHAD, however, there are limitations for a wide application of DHAD in a one-step chemoenzymatic synthesis of 2-keto-3-deoxy-aldonic acids in a preparative scale. Apparently the substitution of the two aliphatic methyl or ethyl groups at C-3 of the natural substrates **3** and **4** by only one polyhydroxy chain, characteristic of aldonic acids causes a drop of the conversion rates for all tested acids **12**, **13**, **15**, **17** and **19**. The principle reason for this phenomenon can be demonstrated to be an electronic destabilisation effect of the initially formed cationic-like intermediate by the electronic withdrawing character of the new hydroxy groups. The results of the inhibitor kinetics with the 3-deoxy aldonic acids **25**, **27** and **29** show binding at the active site of the enzyme for all tested aldonic acids **12**, **13**, **15**, **17**, **19**, **25**, **27** and **29**. This also indicates the second limitation for possible substrate modifications. The ratio between decreasing binding capacity and increasing chain length prohibit a useful application of DHAD for a chemoenzymatic one-step conversion of higher aldonic acids such as aldoheptonic and aldooctonic acids, which would lead to the synthesis of the biologically most relevant 2-keto-3-deoxy-D-*arabino*-heptonic acid and 2-keto-D-*manno*-octonic acid (KDO).

Experimental

Materials

Blender: ESGE AG (M 100); centrifuge: Beckmann J 2-21 and Eppendorf 5415; freeze drying: Leybold–Heraeus Lyovac GT 2; spectrophotometer: Beckmann DU-62; ion exchange resins: DEAE 23SH-cellulose (0.05–0.2 mm, Serva), Lewatit H+ SP 1080 (0.2–0.3 mm, 50–70 mesh, Merck); dialysis: cellulose 12000 M_r cut off (16 mm, Sigma); immobilisation: activated CH Sepharose 4B (Pharmacia); HPLC: pump 64, analytical head, column oven and R_i-detector 88 (Knauer); column HPX 87 (organic acids; Aminex); eluent: H_2O/H_2SO_4 (pH 3);

conditions of elution: T = 35 °C, p = 60–65 bar, flow 0.6 mL min^{-1}; NMR: Brucker AMX 400; ^1H (400 MHz); ^{13}C (100.6 MHz). Melting points are uncorrected. All chemicals were purchased from Merck, Aldrich, Fluka and Sigma.

Enzyme purification

Purification of DHAD from fresh spinach leaves was carried out as described by Pirrung *et al.*[15] with slight modifications. For all solutions bidistilled water was used and all purification steps were carried out at 4 °C.

Homogenisation. Fresh spinach leaves (600 g) obtained from a local market were homogenised in 1.3 L buffer I (0.1 M Tris–HCl; 10 mM $MgCl_2 \cdot 6H_2O$; 1 mM EDTA; pH = 8.00) by several treatments with a blender. The insoluble material was removed by filtration through a cotton cloth and the solution was centrifuged for 20 min.

Polymin P precipitation. The obtained solution was brought to a concentration of 0.2 M NaCl and then 5 mL L^{-1} of a 12.5% aq. solution of polymin P was added dropwise within 15 min. The formed precipitate was removed after 30 min stirring by centrifugation for 15 min.

Ammonium sulphate precipitation. Finely ground ammonium sulphate was added within 20 min up to 30% saturation and stirring was continued for an additional 30 min. The precipitate was removed by centrifugation for 30 min and the solution was again treated with finely ground ammonium sulphate as described above up to 55% degree of saturation. Stirring was continued for 1 h. The suspension was centrifuged for 30 min and the pellet was resuspended in 80 mL buffer II (same composition as buffer I without EDTA). The suspension was dialysed twice with 2 L buffer II and centrifuged for at least 15 min.

DEAE–cellulose chromatography. The solution obtained was subjected to a column (80 cm; diameter 2 cm) filled with DEAE–cellulose equilibrated with buffer II, and eluted by 2 L of a linear gradient from 0 to 0.5 M NaCl in buffer II with a flow rate of 1 mL min^{-1}. The fractions containing DHAD activity were pooled and dialysed twice with 2 L buffer II. For lengthy storage the frozen solution was kept at –20 °C with less than 10% loss of activity over 3 months.

Assays

The amount of protein was detected by the biorad procedure. DHAD activity was measured by two colorimetric assays.[23]

2,4-Dinitrophenylhydrazine (DNPH)-assay. To samples of the protein solutions (10–200 µL) 100 µL of an 80 mM solution of racemic substrate **3** + **3a** in buffer II was added and brought to a final volume of 400 µL by addition of buffer II. After 30 min incubation at 35 °C the reaction was stopped by addition of 100 µL 10% aq. solution of trichloroacetic acid. 200 µL of a saturated solution of DNPH in 2 N HCl were added and the solution was kept for 20 min at rt followed by the addition of 800 µL of a 2.5 N aq. solution of NaOH. After 30 min at rt the solution was degassed by ultrasonification and centrifuged for 5 min. The absorbance was measured at 538 nm and evaluated by a calibration plot employing product **5**.

Semicarbazide (SCA)-assay. The samples were prepared and incubated as described above. The reaction was stopped by addition of 100 µL 2 N aq. HCl. Then 300 µL of a solution containing 1.0 g semicarbazide hydrochloride and 1.5 g sodium acetate per 100 mL bidest. water was added. The solution was kept for 15 min at 30 °C, diluted with 500 µL bidistilled water, degassed by ultrasonification and centrifuged for 5 min. The absorbance was measured at 250 nm and evaluated by a calibration plot employing either product **5** or chemically synthesised compound **10**.

Kinetic studies

For measurement of enzyme kinetics 10 samples of each substrate in a concentration range of 0.1–10 mM were incubated with 50 mU DHAD. The product formation was followed over 30 min for **3** and **12** each 5 min or, respectively, over 90 min for **13** each 15 min by the SCA-assay. The kinetic parameters were evaluated by the method of Hanes. For the inhibitor kinetics 10 samples of the natural substrate **3** in the concentration range of 0.1 to 10 mM were incubated together with each inhibitor **25**, **27** and **29** at five concentrations between 1 and 5 mM. The new kinetic parameters were evaluated as described above and the K_i values were obtained as shown in Scheme 7.

Immobilisation of DHAD

The immobilisation was carried out as described by Parikh *et al.*[20] 2 g Activated CH-Sepharose 4B (Pharmacia) were suspended in 25 mL 5 mM aq. HCl and washed with 400 mL of a buffer containing 0.1 M $NaHCO_3$ and 0.5 M NaCl at pH 8. The gel was treated with 3.6 U DHAD (25 mL 28 mg^{-1} protein) and shaken

fraction	protein (mg)	activity (U)*	specific activity (U mg^{-1})	activity yield (%)	enrichment
crude	1755	54.7	0.03	100	1
Polymin P	1026	47.9	0.05	87.6	1.67
$(NH_4)_2SO_4$	231	24.8	0.11	45.3	3.67
DEAE	30	19.9	0.66	36.4	22

*1 U = formation of 1 µmol product **5** per min.

for 24 h at 4 °C. The suspension was centrifuged and the gel was washed several times with a buffer containing 0.1 M Tris–HCl and 0.5 M NaCl at pH 8. After incubation with this buffer for 4 h the suspension was centrifuged and washed several times with buffer II. The activity of the immobilisate was measured to be 2.47 U, that means 86% yield compared to another sample of 3.6 U DHAD which was kept for 30 h at 4 °C without any manipulations to yield 2.88 U.

Chemical synthesis of substrates and inhibitors

The racemic natural substrate **3** + **3a** was synthesised according the procedure described by Lemmich *et al.*[24] Sodium 2-keto-3-deoxy-D-*threo*-hexonic acid monohydrate was prepared as previously described.[16] Lithium L-threonic acid monohydrate (**12**) and potassium D-erythronic acid monohydrate (**13**) were prepared according to Ref. 25. Potassium L-arabinonic acid monohydrate (**15**) and potassium D-galactonic acid monohydrate (**19**) were synthesised as described by Moore *et al.*[26] Potassium D-gluconic acid monohydrate (**17**) was obtained from Merck.

The 3-deoxy-aldonic acids **25**, **27** and **29** were obtained by titration of the corresponding 3-deoxy-aldono-1,4-lactones with a 0.25 N aq. solution of lithium hydroxide to a pH of 7.4. A typical procedure is described for **25**. The corresponding 3-deoxy-aldono-1,4-lactones were prepared according to the procedure described by Pedersen *et al.*[22]

*2,3-Di-O-acetyl-D-*erythrono-*1,4-lactone (23).* D-*erythrono*-1,4-Lactone (**22**) (6.2 g, 52.5 mmol) was dissolved in acetic acid anhydride (50 mL) and treated with 5 drops of $HClO_4$. The work-up was carried out as described by Pedersen *et al.* [22] to yield **23** (10.36 g, 92%) as a syrup; $[\alpha]_D^{20}$ = –62.3° (*c* 1.00, $CHCl_3$); ^1H NMR ($CDCl_3$): δ 5.45 (*m*, 2H, H-2, H-3), 4.35 (*dd*, 1H, $J_{3,4}$ = 2.5, $J_{4,4'}$ = 11.5 Hz, H-4), 4.25 (*d*, 1H, H-4'), 1.95, 1.90 (*s*, *s*, 3H, 3H, 2OAc); ^{13}C NMR ($CDCl_3$): δ 169.6, 169.2, 168.6 (C-1, 2 $\underline{C}OCH_3$), 69.0 (C-4), 68.4, 66.5 (C-2, C-3), 19.9, 19.6 (2 $CO\underline{C}H_3$).

2(R,S)-Hydroxy-butyro-1,4-lactone (24). Compound **23** (6.06 g, 30 mmol) was dissolved in ethyl acetate (100 mL) and triethylamine (10 mL) and palladium 10% on carbon (500 mg) was added. The solution was stirred for 24 h in a hydrogen atmosphere. The catalyst was filtered off and the solution was treated with KOH (5 g) dissolved in ethanol (50 mL) and water (50 mL). After stirring for 18 h at rt the solution was passed through a column packed with Lewatit cation exchange resin. The fractions containing the product were pooled and freeze-dried to obtain **24** (2.4 g) in 79% yield as a light yellow syrup; ^1H NMR ($CDCl_3$): δ 4.30 (*dd*, 1H, $J_{2,3}$ = 8.0, $J_{2,3'}$ = 10.0 Hz, H-2), 4.18 (*ddd~dt*, 1H, $J_{3,4}$ = 2.0, $J_{3',4}$ = 2.0, $J_{4,4'}$ = 9.0 Hz, H-4); 4.00 (*ddd~dt*, 1H, $J_{3,4'}$ = 6.0, $J_{3',4'}$ = 6.0 Hz, H-4'), 3.9–3.7 (*br*, 1H, OH), 2.35 (*dddd*, 1H, $J_{3,3'}$ = 12.5 Hz, H-3), 2.05 (*m*, 1H, H-3'); ^{13}C NMR ($CDCl_3$): δ 178.4 (C-1), 67.4 (C-2), 65.4 (C-4), 30.9 (C-3). For the determination of the proportions of the single enantiomers **24** was treated with 2(*R*)-methoxy-2-phenyl-3,3,3-trifluoropropionic acid chloride according to the procedure described by Mosher *et al.*[27] The enantiomeric proportion was detected as 1:1 by integration of the ^1H NMR (H-2: 5.67 and 5.55).

Lithium 2(R,S)-4-dihydroxy butyric acid monohydrate (25). Compound **24** (600 mg, 6 mmol) was dissolved in bidist. water (20 mL) and titrated with a 2.5 mM aq. solution of lithium hydroxide to pH 7.4. The solvent was removed by freeze drying to obtain **25** (717 mg) in 95% yield as a white solid; mp 212–220 °C; ^1H NMR (D_2O/CH_3CN): δ 4.15 (*dd*, 1H, $J_{2,3}$ = 4.0, $J_{2,3'}$ = 8.0 Hz, H-2), 3.78 (*ddd*, 1H, $J_{3,4'}$ = 7.0, $J_{3',4'}$ = 6.0, $J_{4,4'}$ = 12.0 Hz, H-4), 3.72 (*ddd*, 1H, $J_{3,4'}$ = 7.5, $J_{3',4'}$ = 7.0 Hz, H-4'), 2.05 (*dddd*, 1H, $J_{3,3'}$ = 14.5 Hz, H-3), 1.85 (*dddd*, 1H, H-3'); ^{13}C NMR (D_2O/CH_3CN): δ 180.4 (C-1), 68.8 (C-2), 57.7 (C-4), 35.5 (C-3). Found: C, 33.35; H, 6.30; calcd for $C_4H_9O_5Li$: C, 33.12; H, 6.26.

3-Desoxy-L-*threo*-pentono-1,4-lactone (**26**) and 3-desoxy-D-*xylo*-hexono-1,4-lactone (**28**) were prepared according to Ref. 22.

*Lithium 3-desoxy-L-*threo*-pentonic acid monohydrate (27).* Compound **26** (500 mg, 3.78 mmol) was subjected to the same procedure as described for **25** to obtain **27** (754 mg) in 97% yield as a white solid; mp 238 °C (dec.); $[\alpha]_D^{20}$ = –20.5° (*c* 1.00, H_2O); ^1H NMR (D_2O/CH_3CN): δ 4.24 (*dd*, 1H, $J_{2,3}$ = 3.0, $J_{2,3'}$ = 10.0 Hz, H-2), 3.97 (*dddd*, 1H, $J_{3,4}$ = 9.5, $J_{3',4}$ = 3.0, $J_{4,5}$ = 4.0, $J_{4,5'}$ = 7.0 Hz, H-4), 3.68 (*dd*, 1H, $J_{5,5'}$ = 12.0 Hz, H-5), 3.57 (*dd*, 1H, H-5'), 1.90 (*ddd*, 1H, $J_{3,3'}$ = 13.0 Hz, H-3), 1.75 (*ddd*, 1H, H-3'); ^{13}C NMR (D_2O/CH_3CN): δ 180.8 (C-1), 68.5, 67.9 (C-2, C-4), 64.9 (C-5), 36.6 (C-3). Found: C, 34.50; H, 6.37; calcd for $C_5H_{11}O_6Li$: C, 34.12; H, 6.35.

*Lithium 3-desoxy-D-*xylo*-hexonic acid monohydrate (29).* Compound **28** (547 mg, 3.38 mmol) was subjected to the procedure described for **25** to obtain **29** (669 mg) in 97% yield as a white solid; mp 194 °C (dec.); $[\alpha]_D^{20}$ = –24.8° (*c* 1.00, H_2O); ^1H NMR (D_2O/CH_3CN): δ 4.27 (*dd*, 1H, $J_{2,3}$ = 3.0, $J_{2,3'}$ = 9.5 Hz, H-2), 3.95 (*ddd~dt*, 1H, $J_{3,4}$ = 10.5, $J_{3',4}$ = 3.0, $J_{4,5}$ = 3.0 Hz, H-4), 3.80 (*dd~t*, 1H, $J_{5,6}$ = 7.5, $J_{6,6'}$ = 7.5 Hz, H-6), 3.70 (*m*, 2H, H-5, H-6'), 2.05 (*ddd*, 1H, $J_{3,3'}$ = 13.5 Hz, H-3), 1.75 (*ddd*, 1H, H-3'); ^{13}C NMR (D_2O/CH_3CN): δ 180.8 (C-1), 73.7 (C-2), 68.5, 67.3 (C-4, C-5), 61.9 (C-6), 36.9 (C-3). Found: C, 35.31; H, 6.42; calcd for $C_6H_{13}O_7Li$: C, 35.59, 6.04.

Chemoenzymatic synthesis

2-Keto-isovaleric acid (5). The racemic natural substrate **3** + **3a** (172 mg, 1 mmol) was dissolved in buffer II and incubated for 24 h at 35 °C together with 2 U DHAD immobilised on activated CH–Sepharose 4B. The gel was filtered off and the solution was subjected to a column filled with Lewatit H^+ cation exchange resin. The solution was concentrated by freeze drying. One tenth of the solid was dissolved in bidistilled water (10 mL) and was separated in several samples by HPLC chromatography on a HPX 87 H column

(Aminex). After freeze drying **5** (6.7 mg) was obtained in 44% yield in addition to re-isolated **3a** (6.4 mg, 0.044 mmol) in 44% yield. The spectroscopic data were in correspondence to that of the commercial compound.

2-Hydroxy-butyro-2-en-1,4-lactone (20). Compound **12** (27.3 mg, 0.172 mmol) was incubated for 3 days at 35 °C as described for **5**. The gel was filtered off and the solution was subjected to a column filled with Lewatit H^+ cation exchange resin. The solution was concentrated by freeze drying to a volume of 3 mL and the product was separated by several extractions with diethylether. Concentration of the organic layer afforded **20** (3.8 mg) in 22% yield as a light yellow solid; mp 105 °C (Ref. 21: 106 °C); ^{1}H NMR ($CDCl_3$): δ 6.23 (*t*, 1H, $J_{3,4}$ = 2.0 Hz, H-3), 4.62 (*d*, 2H, H-4, H-4'); ^{13}C NMR ($CDCl_3$): δ 171.1 (C-1), 141.4 (C-2), 114.0 (C-3), 67.2 (C-4).

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