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Effects of Chirality and Substituents at Carbon 3 in Dihydroxyacetone-phosphate Analogues on their Binding to Rabbit Muscle Aldolase

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Abstract—A series of dihydroxyacetone-phosphate (DHAP) analogues has been synthesized, differing in their stereochemistry and functionality at C-3. The kinetic effects of these compounds on the enzyme aldolase (EC 4.1.2.13) have been studied and differing modes of action observed. Competitive and time dependant reversible inhibition have been shown to take place both with and without borohydride detected formation of an immonium ion.

Introduction

Rabbit muscle aldolase catalyses the reversible cleavage of fructose-1,6-diphosphate (FDP) in two trioses, dihydroxyacetone-phosphate and D-glyceraldehyde-3-phosphate (GAP).[1] In the FDP synthesis direction, the reaction has the following features: (i) Schiff's base formation between DHAP and a lysyl residue at the active site (Lys 229);[2] (ii) pro-*S* proton abstraction at C-3[3] in the immonium leading to an enamine[4,5] whose condensation with GAP gives FDP with an *S* configuration at C-3 (Scheme 1).

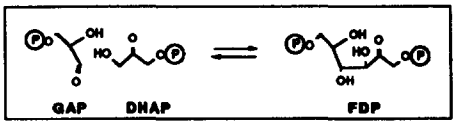
This enzyme, currently in use for synthetic purposes in organic chemistry, accepts a large variety of aldehydes as substrates, but is rather selective toward DHAP since only limited modifications at C-1 are accepted.[6] Aldolase is also selective with regard to chirality at C-3: L-erythrulose-1-phosphate[7] and D-xylulose-1,5-diphosphate,[8] both with *S* configuration at C-3, are cleaved into DHAP and formaldehyde or glycoaldehyde, respectively. In contrast, D-erythrulose-1-phosphate and D-ribulose-1,5-diphosphate (*R* configuration at C-3) are, respectively, slow-binding[9] and competitive[10] inhibitors of the enzyme. According to Ferroni *et al.*[9] and Rose and Warms,[10] Schiff's base formation is only observed in the first case.

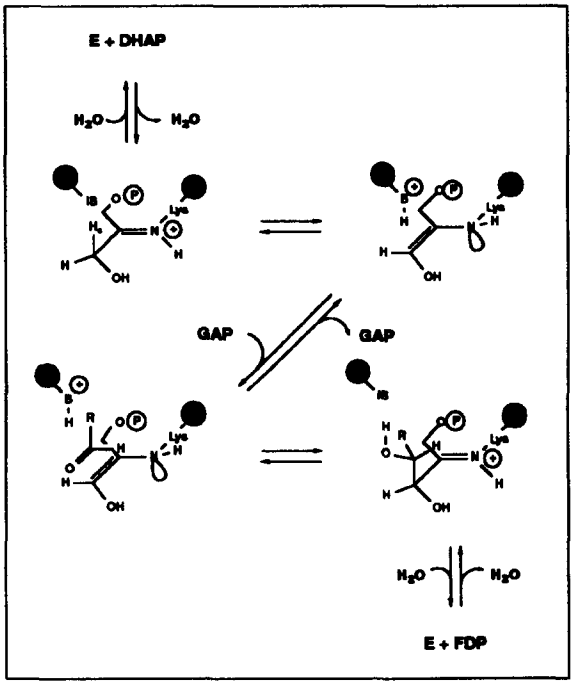
In order to obtain a better insight of the DHAP binding site and to facilitate the understanding of the different situations associated with the structure of the DHAP–enzyme complex at the C-3 locus previously described, we synthesized compounds analogous to DHAP according to Scheme 2. Their interactions with aldolase in terms of affinity constants and abilities to form Schiff's base were investigated.

Results indicate that these features depend on the configuration and the substitution at C-3 of the analogues; the possible role of the hydroxyl group in the reaction mechanism is also discussed.

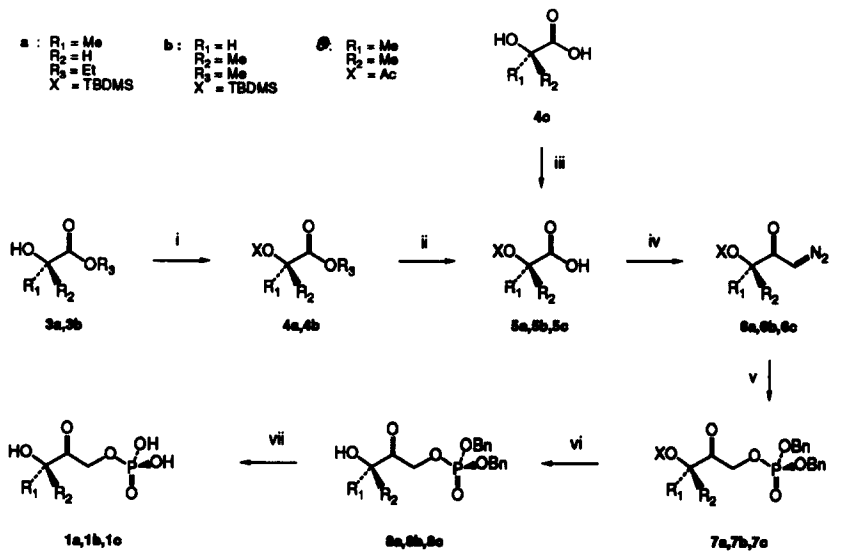
Results

Incubation of aldolase with compounds **1a** (*S* configuration at C-3) and **1b** (*R* configuration at C-3) at





Scheme 1. Reaction catalyzed by aldolase and mechanistic pathway.



i: TBDMSCl, imidazole; ii: (1) NaOH (2) HCl; iii: Ac_2O, pyridine; iv: (1) $(COCl)_2$ (2) CH_2N_2; v: $(BnO)_2P(O)OH$; vi: H_2O, Dowex H^+ (**7a, b**) or H_2O, H_2SO_4 (**7c**); vii: H_2, Pd/C.

Scheme 2. Synthetic scheme for the synthesis of compounds **1a–c**.

concentrations 0.1–5 mM at 25 °C, leads within approximately 15 min to an inhibition plateau. In contrast, compound **1c** produces no effect even at high concentrations (Table 1). The inactivation reaches values of 48 and 70% saturation at concentrations of 5 and 3 mM for compounds **1a** and **b**, respectively. Protection against inactivation by DHAP indicates that inhibition is likely to occur at the active site. The reversibility of inhibition is evidenced by the mixing of an aliquot of the enzyme-inhibitor solution to an assay FDP in saturating conditions: upon this dilution effect, the enzymatic activity is slowly but fully restored (Fig. 1). This observation of time dependent reversible inhibition indicates that compounds **1a** and **b** behave as slow-binding inhibitors of aldolase.[11]

Determination of the kinetic parameters associated with the inhibition of aldolase by compounds 1a and b

The shape of the plots of inhibition reversal illustrated in Figure 1 can be described by the integrated form of the Frieden's equation[12] (see equation 1), which gives the product concentration versus time in the slow-binding inhibition conditions for a first-order process. Data analysis for the restoration of activity upon dilution of the inhibitor allows the determination of the first-order rate constant k_{-2} (Table 2). The dissociation constant K_d of the rapidly formed EI complex and the constant K corresponding to the equilibrium between EI and EI* are obtained from data in Table 1 using equation 2 (see Fig. 2 and Table 2). The first order rate constant k_2 is obtained from K and k_{-2} (Table 2).

Table 1. Aldolase inactivation by compounds **1a–c**

Compound	Concentration (mM)	DHAP (mM)	Initial velocity for remaining activity (%)
1a	5	0	52
	1	0	63
	.5	0	70
	.1	0	90
	.5	1	100
1b	3	0	30
	.5	0	40
	.25	0	45
	.1	0	60
	.25	1	100
1c	8	0	100

Aldolase (0.20 mg mL^{-1}) was incubated in TEA buffer pH 7.6, at 25 °C with compounds **1a**, **b** or **c** in the presence or absence of DHAP at indicated concentrations. After 30 min incubation, the enzyme's initial velocity was measured on 10 µL aliquots. A control experiment was made with the enzyme alone.

Schiff's base formation between aldolase and compounds 1a–c

The ability of compounds **1a–c** to form a Schiff's base was determined by sodium borohydride reduction of

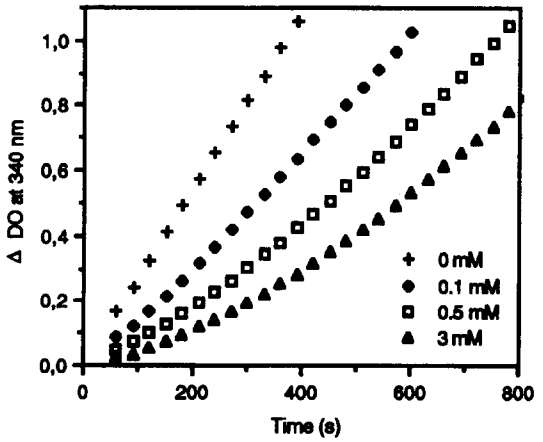


Figure 1. Reversion of aldolase inactivation by **1b** in the presence of FDP. Aldolase was inactivated as shown in Table 1 by compound **1b** at the indicated concentrations. Reversal of inactivation was determined on 10 µL aliquots diluted in 1 mL of TEA buffer containing 1 mM FDP (see Experimental). The same kind of plot was obtained with **1a** (results not shown). The rate value extrapolated to infinite time was found to be identical to that of the reference assay.

enzyme–inhibitor complex solutions in non-saturating conditions (Table 3). Evidence for the occurrence of this reaction is given by the irreversible loss of activity in the case of compounds **1a** and **b** (no restoration of activity upon dilution). Under the same conditions, no change is observed with compound **1c**. The results shown in Table 3 indicate that aldolase inactivation by compounds **1a** and **b** parallels sodium borohydride irreversible inactivation. A higher efficiency is obtained for compound **1b** (see relative concentrations in inhibitor). This means that the Schiff's base is part of (or corresponds totally to) the EI* complex.

Competitive inhibition of aldolase by compounds 1c and 2 (3-hydroxy propane-1-phosphate)

Double reciprocal plots[13] (not shown) of the initial velocities of aldolase for different substrate and inhibitor (**1c** and **2**) concentrations indicate that these compounds are competitive inhibitors, K_i = 4.0 and 4.8 mM, respectively.

Discussion

The comparison between the results obtained with the four investigated inhibitors and also with other results from the literature, allows us to glean additional information concerning the DHAP binding site in aldolase. From available data concerning dissociation and inhibition constants in DHAP analogues, it is apparent that the three functional groups (phosphate, carbonyl and hydroxyl) in DHAP synergistically contribute to their affinity for aldolase. This affinity does not rely particularly on one of the three groups but is dramatically reduced when one is absent, as shown by the similar affinity constants for acetol phosphate (6.7 mM),[14] propane-1-phosphate (13 mM),[15] dihydroxyacetone-sulfate (3 mM)[16] and 3-hydroxypropane-1-phosphate (4.8 mM, compound **2**). This also accounts for the high affinity of phosphoglycolohydroxamic acid (6 μM in 20 mM TEA buffer)[17] despite the absence of Schiff's base formation with this compound.

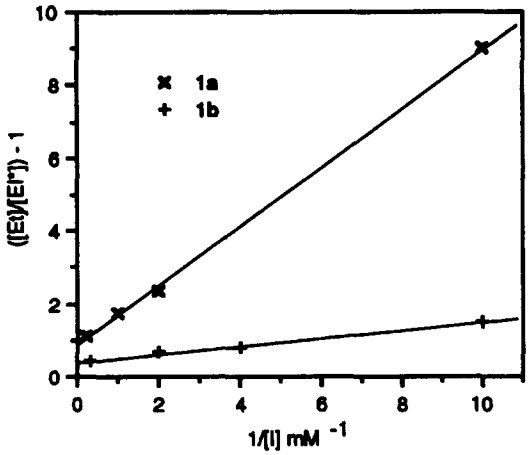


Figure 2. Data treatment of aldolase inhibition values by **1a** and **b** according to equation 2.

Table 2. Kinetic paramaters for aldolase inhibition by compounds **1a** and **b**

Constantes	1a	1b
k-2 (min-1)	0.10	0.11
KI (μM)	800	240
K	0.95	0.45
k2 (min-1)	0.105	0.245
KI* (μM)	390	74.5

Table 3. Aldolase inactivation by sodium borohydride in the presence of compounds **1a–c**

Compound	Concentration mM	Remaining activity % a	b
DHAP	0.25	100	30
	2	100	20
1a	2	60	62
1b	0.25	50	60
	0.5	43	50
1c	6	100	100

a: Incubation without $NaBH_4$ addition.
b: Incubation with $NaBH_4$ addition.
Assay solutions (0.2 mL Tris buffer pH 6.0) contained aldolase (1 mg), inhibitor or DHAP at concentrations indicated in the table. After 30 min, reduction was performed by addition of 5 μL of a solution 0.25 M in $NaBH_4$ in 1 mM sodium hydroxide; 10 min after sodium borohydride addition, samples were withdrawn for measurement of the enzyme activity. In controls, where neither inhibitor nor DHAP had been added, no inactivation was observed after sodium borohydride addition.

The affinity of the aldolase inhibitors investigated in this work is also dependent on steric effects at C-3. However, bulkiness at this position can only be increased to a certain limit: substitution with a phenyl group instead of a methyl moiety (results not shown) suppresses inhibition in both configurations (K_i > 10 mM), whereas the *gem*-dimethyl substituted compound is a weak competitive inhibitor (K_i = 4.0 mM). The affinities of compounds **1a** and **b** for aldolase are also dependent on this chirality at C-3. It is noteworthy that although aldolase only transforms compounds of *S* configuration at C-3, compounds of opposite configuration have similar or even higher affinities. Compounds **1a** and **b**, which contain only a slight modification with regard to DHAP, behave as slow-binding inhibitors. The better inhibition obtained with **1b** can be ascribed to two factors: a higher affinity for the enzyme (K_d value) and a more favourable equilibrium constant for the formation of the EI* complex. For compound **1b**, it is

significant to compare the corresponding inhibition value K_i^* (75 μM) to the K_m value for DHAP (50 μM) and also to the first-order rate constant k_2 (0.24 min^{-1}) to that measured in the same conditions for D-erythrulose-1-phosphate (0.25 min^{-1}), also described as a slow-binding inhibitor.[9]

The protection of aldolase by DHAP against inactivation as well as sodium borohydride reductions indicate that **1a** and **b** bind at the active site and lead to the formation of the Schiff's base (the reduction of which produces an irreversible inactivation of the enzyme). The larger amount of Schiff's base formed with **1b** compared to **1a** indicates a more favourable formation and protonation of the carbinolamine intermediate. The larger K and k_2 values obtained with compound **1b** compared to those for compound **1a** can also be accounted for, besides the larger amount of Schiff base, by the possible enamine formation since compound **1b** has a correctly oriented proton.[3] However, evidence for this possible enamine contribution could not be given experimentally by the enzymatic synthesis product since the racemic mixture **1a** + **b** does not lead to any condensation product with GAP in the presence of aldolase.[6] Results also confirm the importance of the OH group at C-3 on the stabilization of the immonium. Acetol-phosphate weakly forms an immonium,[18] whereas **1a** and **b**, bearing an OH group do form it, and to a larger extent in **1b** where the OH group is properly oriented for enamine formation.

Concerning the origin of the slow-binding effect, the kinetic analysis itself does not allow any rationalization, particularly, the importance of the conformational changes of the enzyme during inhibitor binding and debinding has not been determined. We can, however, refer to the importance of the conformational changes of aldolase since the rate determining steps in both synthesis and cleavage reactions correspond to product release.[19] This proposal of conformational change induced by **1a** and **b** is supported by a work from Kochman *et al.*[20] where it is shown that monophosphorylated compounds induce different conformational changes to those induced by diphosphorylated ones. It is noteworthy that slow-binding effects are observed with monophosphorylated compounds such as **1a**, **b** or D-erythrulose-1-phosphate, whereas diphosphorylated substrate analogues such as D-ribulose-1,5-diphosphate[10] or 4-deoxy-fructose-1,6-diphosphate[21] produce competitive inhibition.

Finally, on the grounds of the results presented in this work and from other sources in the literature, it can be seen that aldolase offers favourable features for slow-binding inhibition by DHAP analogues bearing the structural requirements discussed in this paper. This may be of interest for the rational design of active compounds against parasites where glucose metabolism is essential.[21]

Experimental

Enzymes and reagents

FDP sodium salt, DHAP lithium salt, glycerol phosphate dehydrogenase, triose-phosphate isomerase and rabbit muscle aldolase were purchased from Boerhinger Mannheim. All other chemicals were purchased from Aldrich and were used without extra purification.

Assay methods

Aldolase activity (10 units mg^{-1} at 25 °C) was measured by means of the triose-phosphate isomerase/glycerol-1-phosphate dehydrogenase method in 1 mL of 0.1 M triethanolamine buffer (HCl, 1 mM EDTA, pH 7.6 and ionic stength 0.15), using FDP (1 mM) as substrate.[22] The initial rates were calculated from the absorbance change of NADH ($\varepsilon = 6.22$ mM^{-1} cm^{-1}) at 340 nm with a Lambda 15 Perkin Elmer spectrophotometer. The protein concentration was estimated using $A^{1\%}$ (280 nm) = 9.1.[23]

Inhibition study

Aldolase (0.2 mg mL^{-1} in 0.2 mL TEA buffer) was incubated over a period of maximum 30 min in the presence of the compound under study (0.1–6 mM). The enzymatic activity was assayed as a function of time with 10 μL aliquots. Control experiments were run without inhibitor and all measurements were made in triplicate. Plots of the restoration of enzymatic activity were analyzed using the VA04A program.[24]

Competitive inhibition study

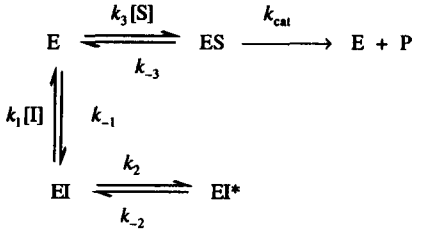
The dissociation constants of enzyme–inhibitor complexes were determined by double reciprocal plots of the initial velocities of aldolase (0.002 mg mL^{-1}) for FDP and inhibitor concentrations in the range of 10–100 μM and 3–6 mM, respectively.

Sodium borohydride reductions

Reductions were performed according to the previously described technique.[16]

Equations

For the general system:



P production versus time is given by :

$$P = V_s t + (V_o - V_s).(1 - e^{-k't})/k', \quad (1)$$

where V_o, V_s and k' represent the initial velocity, the steady-state rate and the apparent first-order rate constant for reaching the equilibrium between EI and EI*, respectively.[11]

For this system:

$$k' = k_{-2} + k_2\left(\frac{[I]/K_d}{1+[S]/K_S+[I]/K_d}\right),$$

where K_d, K_S, S and I represent the dissociation constant of the EI complex, the Michaelis constant for the substrate, the substrate concentration and the inhibitor concentration respectively (after dilution, for inhibitor concentration, close to zero, $k' = k_{-2}$).

For the equilibrium constants associated with the formation of EI and EI* (without the substrate S), they are defined as follows:

$$[EI^*] = \frac{[E_t]}{K(1+K_d/[I])+1} \qquad K_d = \frac{k_{-1}}{k_1}$$

$$K = \frac{k_{-2}}{k_2}$$

$$\frac{[E_t]}{[EI^*]} - 1 = K + \frac{KK_d}{[I]}, \quad (2) \qquad K_i^* = \frac{K_d k_{-2}}{k_2 + k_{-2}}$$

where $\frac{[E_t]}{[EI^*]}$ is the reciprocal of inactivated enzyme.

Synthesis

NMR spectra were recorded on a Bruker apparatus AC200 (200 MHz ^{1}H NMR, 80 MHz ^{31}P NMR and 50 MHz ^{13}C NMR) or a Bruker AC80 (80 MHz ^{1}H NMR) spectrophotomer. Chemical shifts, δ, are reported in ppm relative to the deuteriated solvent used. Elemental analyses were performed by the Ecole Nationale Supérieure de Chimie de Toulouse. Optical rotations were measured with a Perkin–Elmer 241 polarimeter at room temperature.

General procedure for the reaction with diazomethane of protected hydroxy-acids 5a, b or c

Protected acids **5a**, **b** or **c** (5–10 mmol) were dissolved in 10–15 mL dry benzene. Approximately 5 mL of solvent was removed by distillation to eliminate water. Two equivalents of oxalyl chloride were added dropwise at room temperature and the reaction mixture was stirred for 2 h. The progress of the reaction was followed by means of IR spectrophotometry. Excess oxalyl chloride and most of the solvent was removed by distillation. The remaining solution, diluted in 10–15 mL of dry Et_2O, was added dropwise at –20 °C to a solution of CH_2N_2 in Et_2O (3 equivalents in 70–140 mL of Et_2O prepared according the protocol of the manufacturer). After addition, the reaction mixture was stirred at room temperature over 2 h, and the progress of the reaction was again followed by IR spectrometry. Excess CH_2N_2 was removed by purging with Ar and destroyed in HOAc solution. The solvent was removed by evaporation *in vacuo* and the remaining residue provided, after chromatography (silica gel, Et_2O hexane, 1:5–2:5), diazoketone **6a**, **b** or **c** as an unstable slightly yellow oil.

General procedure for phosphorylation of diazoketones 6a, b or c

A mixture of diazoketone **6a**, **b** or **c** (2–8 mmol in 30–50 mL dry benzene) and dibenzylphosphate (1.1 equivalent) was stirred at 60 °C for 8–12 h. The progress of the reaction was followed by IR spectrophotometry and TLC (hexane:EtOAc:CH_2Cl_2, 1:1:1). Solvent was removed by evaporation *in vacuo* and the crude residue, after chromatography (silica gel, hexane:EtOAc:CH_2Cl_2, 2:1:1–1:1:1) yielded the corresponding phosphorylated compounds **7a**, **b** or **c** as colourless oils.

General procedure for hydrogenolysis of dibenzylphosphorylated compounds 7a–c

A mixture of protected phosphate **7a**, **b** or **c** (about 0.5 mmol in 10 mL EtOH) and 100 mg 10% Pd on charcoal was hydrogenated over a period of 4 h. The catalyst was eliminated by filtration and washed copiously with MeOH. Freshly distilled cyclohexylamine (2.1 equivalents) was added, and the solvent removed by evaporation *in vacuo* to yield a white powder. The powder was washed twice with Et_2O (2 × 3 mL) and dried *in vacuo*.

*(S)-Ethyl-2-((*tert*-butyldimethylsilyl)oxy) propionate (4a).* To a mixture of (*S*)-ethyllactate (**3a**) (5.31 g, 45 mmol) and imidazole (9 g, 132 mmol) was added *tert*-butyldimethylsilyl chloride (8 g, 53 mmol). The mixture was stirred overnight at 40 °C and extracted with Et_2O (3 × 50 mL). The solvent was removed by evaporation *in vacuo* and the crude residue, after flash distillation (1 torr, 100 °C) yielded compound **4a** as a colourless liquid (9.6 g, 91.9%). $[\alpha]^{20}_D$ –1.17° (*c* 5.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 80 MHz): δ 0.025 (*s*, 3H), 0.05 (*s*, 3H), 0.86 (*s*, 9H), 1.27 (*d*, *J* = 6.3 Hz, 3H), 1.30 (*t*, *J* = 6.4 Hz, 3H), 4.10 (*q*, *J* = 6.4 Hz, 2H), 4.20 (*q*, *J* = 6.3 Hz, 1H). Anal. Calcd for $C_{11}H_{24}O_3Si$: C, 56.85; H, 10.41. Found: C, 56.71; H, 10.60.

*(S)-2-((*tert*-Butyldimethylsilyl)oxy)propanoic acid (5a).* To a solution of compound **4a** (3 g, 12.9 mmol) in 7.5 mL THF at 0 °C was added dropwise a solution of KOH (0.732 g, 13 mmol) in 1.5 mL MeOH and 3 mL H_2O; the mixture was stirred for 3 h at room temperature. The solvents were removed by evaporation *in vacuo* and the residue co-evaporated three times with absolute EtOH (15 mL). The resulting white solid was washed with Et_2O (3 × 10 mL) before being dissolved in 15 mL H_2O. HCl (1 equivalent in 5 mL H_2O) was added at 0°C and the aqueous phase extracted with CH_2Cl_2 (3 × 50 mL). The combined organic layers were washed with brine (30 mL), then H_2O (30 mL), dried over Na_2SO_4 and evaporated *in vacuo* to give **5a** as a colourless oil (2.14

g, 81.3%). $[\alpha]^{20}_D$ –0.08° (*c* 5.0, $CHCl_3$); ^1H NMR ($CDCl_3$, 80 MHz): δ 0.14 (*s*, 6H), 0.89 (*s*, 9H), 1.46 (*d*, *J* = 6.8 Hz, 1H), 4.37 (*q*, *J* = 6.8 Hz, 1H), 9.65 (*s*, 1H). IR (neat): 3200, 1731.5 cm^{-1}. Anal. Calcd for $C_9H_{20}O_3Si$: C, 52.90; H, 9.86. Found : C, 52.60; H, 10.01.

2-Methyl-2-acetoxy-propanoic acid (5c). A mixture of hydroxy-isobutyric acid **4c** (5.2 g, 50 mmol), acetic anhydride (12 mL, 0.23 mol) in 70 mL dry pyridine was stirred overnight at room temperature. The solvent was removed by evaporation *in vacuo* and the residue was dissolved in 100 mL CH_2Cl_2. The organic phase was washed twice with a saturated solution of sodium bicarbonate (50 mL), dried over Na_2SO_4 and evaporated *in vacuo*. The remaining product was purified by flash distillation (0.4 torr, 150 °C) to yield **5c** as a colourless oil which crystallizes on standing (7.3 g, 75.3%). ^1H NMR ($CDCl_3$, 80 MHz): δ 1.56 (*s*, 6H), 2.04 (*s*, 3H), 11.75 (*s*, 1H). IR (neat): 3200, 1736 cm^{-1}.

(S)-3-((tert-Butyldimethylsilyl)oxy)-1-diazo-2-butanone (6a). According to the general procedure for CH_2N_2 reaction, compound **5a** (1.16 g, 5.7 mmol) yielded **6a** as a colourless oil (1.02 g, 89.5%). ^1H NMR ($CDCl_3$, 80 MHz): δ 0.01 (*s*, 6H), 0.84 (*s*, 9H), 1.24 (*d*, *J* = 6.7 Hz, 3H), 4.14 (*q*, *J* = 6.7 Hz, 1H), 5.87 (*s*, 1H). IR (neat): 2106, 1640 cm^{-1}.

3-Methyl-3-acetoxy-1-diazo-2-butanone (6c). According to the general procedure, compound **5c** (1.10 g, 7.5 mmol) yielded **6c** (1.10 g, 87%). ^1H NMR ($CDCl_3$, 80 MHz): δ 1.45 (*s*, 6H), 2.01 (*s*, 3H), 5.85 (s, 1H). IR (neat): 2108, 1737 cm^{-1}.

(S)-Dibenzyl 3-((tert-butyldimethyldimethyl)oxy)-2-oxo-1-butyl phosphate (7a). According to the general procedure for phosphorylation, compound **6a** (0.53 g, 2.65 mmol) yielded **7a** as a colourless oil (0.613 g, 48%). $[\alpha]^{20}_D$ –0.18° (*c* 5.0, $CHCl_3$). IR (neat): 1745, 1260, 1019 cm^{-1}. ^1H NMR ($CDCl_3$, 200 MHz): δ 0.054 (*s*, 3H), 0.067 (*s*, 3H), 0.67 (*s*, 9H), 1.29 (*d*, *J* = 6.8 Hz, 3H), 4.25 (*q*, $^3J_{HP}$ = 6.8 Hz, 1H), 4.90 (*m*, 2H), 5.10 (*d*, $^3J_{HP}$ = 6.0 Hz, 4H), 7.32 (*s*, 10H). ^{13}C NMR ($CDCl_3$, 50 MHz): δ –5.2, –4.5, 17.9, 25.7, 66.8, 69.6, 74.0, 128, 128.6, 135.8, 206.1. ^{31}P NMR ($CDCl_3$, 80 MHz): δ –0.79. Anal. Calcd for $C_{24}H_{35}O_6PSi$: C, 60.23; H, 7.37. Found: C, 59.90; H, 7.55.

Dibenzyl-3-methyl-3-acetoxy-2-oxo-1-butyl phosphate (7c). According to the general procedure for phosphorylation, compound **6c** (1.00 g, 5.90 mmol) yielded **7c** as a colourless oil (2.10 g, 84.9%). IR (neat): 1736, 1260, 1022 cm^{-1}. ^1H NMR ($CDCl_3$, 80 MHz): δ 1.47 (*s*, 6H), 2.02 (*s*, 3H), 4.75 (*d*, $^3J_{HP}$ = 9.9 Hz, 2H), 5.09 (*d*, $^3J_{HP}$ = 8.0 Hz, 4H), 7.33 (*s*, 10H). ^{13}C NMR ($CDCl_3$, 50 MHz): δ 21.1, 23.5, 67.1, 69.7, 82.6, 118.1, 128.6, 131.6, 170.5, 201.7. ^{31}P NMR ($CDCl_3$, 80 MHz): δ –0.95. Anal. Calcd for $C_{21}H_{25}O_7P$: C, 59.99; H, 5.99. Found : C, 59.43; H, 6.19.

(S)-Dibenzyl-3-hydroxy-2-oxo-1-butyl phosphate (8a). Compound **7a** (0.50 g, 10.4 mmol) in 15 mL MeOH was treated with an ion exchange resin (Dowex 50WX8, H^+ form, 10 equivalents) for 5–10 h at room temperature. The progress of the reaction was followed by TLC (CH_2Cl_2:EtOAc, 1:1). The resin was removed by filtration and washed copiously with MeOH and the filtrate was evaporated *in vacuo*. The remaining colourless oil was purified by chromatography (silica gel, CH_2Cl_2:EtOAc 2:1–1:3) to yield **8a** as a colourless oil (0.21 g, 55.5%). $[\alpha]^{20}_D$ –0.317° (*c* 5.0, $CHCl_3$). IR (neat): 3375, 1741, 1263, 1014 cm^{-1}. ^1H NMR ($CDCl_3$, 80 MHz): δ 1.35 (*d*, *J* = 6.9 Hz, 3H), 3.50 (*br*, 1H, OH), 4.28 (*q*, *J* = 6.9 Hz, 1H), 4.81 (*d*, $^3J_{HP}$ = 10.0 Hz, 2H), 5.10 (*d*, $^3J_{HP}$ = 8.2 Hz, 4H), 7.33 (*s*, 10H). ^{13}C NMR ($CDCl_3$, 50 MHz): δ 19.6, 66.7, 69.9, 71.5, 128.1, 128.7, 135.5, 206.9. ^{31}P NMR ($CDCl_3$, 80 MHz): δ –0.80. Anal. Calcd for $C_{18}H_{21}O_6P$: C, 59.34; H, 5.81. Found: C, 59.01; H, 5.98.

Dibenzyl-3-hydroxy-3-methyl-2-oxo-1-butyl phosphate (8c). A solution of compound **7c** (1.00 g, 2.3 mmol) in 15 mL MeOH was treated with H_2SO_4 (100 μL) and the mixture stirred at 60 °C for 8 h. The progress of the reaction was followed by TLC (CH_2Cl_2:EtOAc, 1:1). After neutralization by a saturated solution of sodium bicarbonate, the solvent was evaporated *in vacuo*. The residue was purified by chromatography (silica gel, CH_2Cl_2:EtOAc, 2:1–1:3) to yield **8c** as a colourless oil (0.404 g, 44.9%). IR (neat) : 3378, 1737, 1258, 1023 cm^{-1}. ^1H NMR ($CDCl_3$, 80 MHz): δ 1.32 (*s*, 6H), 3.81 (*s*, 1H, OH), 4.99 (*d*, $^3J_{HP}$ = 10.5 Hz, 2H), 5.09 (*d*, $^3J_{HP}$ = 8.0 Hz, 4H), 7.32 (*s*, 10H). ^{13}C NMR ($CDCl_3$, 50 MHz): δ 26.7, 68.1, 69.1, 76.5, 128.1, 128.7, 135.6, 208.2. ^{31}P NMR ($CDCl_3$, 80 MHz): δ –1.06. Anal. Calcd for $C_{19}H_{23}O_6P$: C, 60.31; H, 6.13. Found: C, 59.45; H, 6.34.

(S)-3-Hydroxy-2-oxo-1-butyl phosphate (1a). According to the general procedure for hydrogenolysis, compound **8a** (0.200 g, 0.55 mmol) yielded **1a** as a biscyclohexylammonium salt (0.190 g, 90.4%). Acid form: $[\alpha]^{20}_D$ –0.22° (*c* 5.0, MeOH). IR (neat): 3370, 1740, 1263, 1013 cm^{-1}. ^1H NMR (CD_3OD, 80 MHz): δ 1.35 (*d*, *J* = 6.8 Hz, 3H), 4.30 (*q*, *J* = 6.8 Hz, 1H), 4.78 (*d*, *J* = 8.7 Hz, 2H). ^{13}C NMR (CD_3OD, 50 MHz): δ 17.5, 66.0, 69.6, 206.3. ^{31}P NMR (CD_3OD, 80 MHz): δ 2.68. Anal. Calcd for $C_{16}H_{35}O_6N_2P$: C, 50.25; H, 9.22. Found: C, 49.95; H, 9.35.

3-Hydroxy-3-methyl-2-oxo-1-butyl phosphate (1c). According to the general procedure for hydrogenolysis, compound **8c** (0.200 g, 0.52 mmol) yielded **1c** as a biscyclohexylammonium salt (0.186 g, 90.2%). IR (KBr): 1732, 1260, 1042 cm^{-1}. ^1H NMR acid form (D_2O, 80 MHz): δ 1.33 (*s*, 6H), 4.94 (*d*, *J* = 6.5 Hz, 2H). ^{13}C NMR (D_2O, 50 MHz): δ 25.5, 26.0, 27.1, 27.1, 32.1, 51.3, 67.6, 77.6, 214.5. ^{31}P NMR (D_2O, 80 MHz): δ 3.73. Anal. Calcd for $C_{17}H_{37}O_6N_2P$: C, 51.50; H, 9.41. Found: C, 51.25; H, 9.55.

3-Hydroxy propane-1-phosphate (2). Synthesis of compound **2** was performed according to the procedure of Khorana *et al.*[25] Compound **2**, barium salt: ^1H NMR

(D_2O, 200 MHz): δ 1.78 (*tt*, *J* = 6.4 Hz, 2H), 3.70 (*t*, *J* = 6.4 Hz, 2H), 3.80 (*td*, *J* = 6.4 Hz, $^3J_{HP}$ = 6.2 Hz, 2H). ^{13}C NMR (D_2O, 50 MHz): δ 35.1, 61.3, 63.8. ^{31}P NMR (D_2O, 80 MHz): δ 4.75.

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References

1. (a) Horecker, B. L.; Tsolas, O.; Lai, C. Y. In *The Enzymes*, 3rd ed.; Boyer, P. D., Ed.; Academic Press: New York, 1972; Vol. VII, p. 213; (b) Meyerhoff, O.; Lohmann, K.; Shuster, P. *Biochem. Z.* **1936**, *286*, 319

2. (a) Grazi, E.; Cheng, T.; Horecker, B. L. *Biochem. Biophys. Res. Commun.* **1962**, *7*, 250; (b) Lai, C. Y.; Nakai, N.; Chang, D. *Science* **1974**, *183*, 1204.

3. (a) Rose, I. A.; Rieder, S. V. *J. Am. Chem. Soc.* **1955**, *77*, 5764; (b) Rose, I. A. *J. Am. Chem. Soc.* **1958**, *80*, 5835.

4. Christen, P.; Riordan, J. F. *Biochemistry* **1968**, *7*, 1531.

5. Riordan, J. F.; Christen, P. *Biochemistry* **1969**, *8*, 2381.

6. Bednarski, M. D.; Simon, E. S.; Bischofberger, N; Fessner, V. D.; Kim, M. J.; Lees, W.; Saito, T.; Waldmann, H.; Whitesides, G. M. *J. Am. Chem. Soc.* **1989**, *111*, 627.

7. Gillet, J. W.; Ballou, C. E. *Biochemistry* **1963**, *2*, 547.

8. Mehler, A. H.; Cusic, M. E. *Science* **1967**, *155*, 1101.

9. Ferroni, E. L.; Harper, E. D.; Fife, W. K. *Biochem. Biophys. Res. Commun.* **1991**, *176*, 511.

10. Rose, I. A.; Warms, J. V. B. *Biochemistry* **1985**, *24*, 3952.

11. (a) Willams, J. W. W.; Morrison, J. F. *Meth. Enzymol.* **1979**, *63*, 437; (b) Morrison, J. F.; Walsh, C. T. In *Adv. Enzymol.*; Meister, A. Ed.; Interscience: New York, 1988; Vol. 61, pp. 201–300.

12. Frieden, C. *J. Biol. Chem.* **1970**, *245*, 5788.

13. Segel, I. H. *Enzyme Kinetics*; Wiley-Interscience: New York, 1975.

14. Rose, I. A.; O'Connell, E. L. *J. Biol. Chem.* **1969**, *244*, 126.

15. Ogata, H.; Fukada, T.; Yamamoto, K.; Funakoshi, J.; Takada, K.; Yasue, N.; Fujisaki, S.; Kajigaeshi, S. *Biochem. Biophys. Acta* **1992**, *1119*, 123.

16. Grazi, E.; Sivieri-Pecorari, C.; Gagliano, R.; Trombetta, G. *Biochemistry* **1973**, *12*, 2583.

17. Lewis, D. J.; Lowe, G. *Eur. J. Biochem.* **1977**, *80*, 119.

18. Pratt R. F. *Biochemistry* **1977**, *16*, 3988.

19. Rose, I. A.; Warms, J. V. D.; Kuo, D. J. *J. Biol. Chem.* **1987**, *262*, 692.

20. (a) Heyduck, T.; Michalczyk, R.; Kochman, M. *J. Biol. Chem.* **1991**, *266*, 15650; (b) Kochman, M.; Dobryszycki, P. *Acta Biochim. Polon.* **1991**, *38*, 407.

21. Perié, J.; Riviere-Alric, L; Blonski, C.; Gefflaut, T.; Lauth de Viguerie, N.; Trinquier, M.; Willson, M.; Opperdoes, F. R.; Callens, M. *Pharmac. Ther.* **1993**, *60*, 347.

22. Misset, O.; Opperdoes, F. R. *Eur. J. Biochem.* **1984**, *144*, 475.

23. Baranowski, T.; Niederland, T. R. *J. Biol. Chem.* **1949**, *180*, 543.

24. Andre, P. Thesis, Université Paul Sabatier, Toulouse, France, n° d'ordre 1693, 1975.

25. Khorana, H. G.; Tener, G. M.; Wright, R. S.; Moffatt, J. G. *J. Am. Chem. Soc.* **1957**, *79*, 430.

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