Pergamon

Bioorganic & Medicinal Chemistry, Vol. 4, No. 12, pp 2043–2054, 1996

PII: S0968-0896(96)00221-0
0968-0896/96 $15.00+0.00

Slow Reversible Inhibitions of Rabbit Muscle Aldolase with Substrate Analogues: Synthesis, Enzymatic Kinetics and UV Difference Spectroscopy Studies

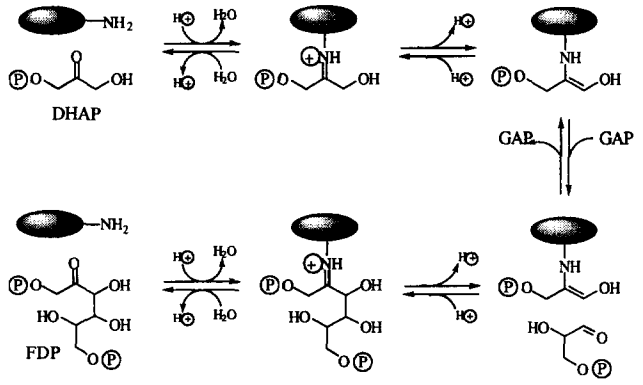
T. Gefflaut, C. Blonski and J. Périé*

Groupe de Chimie Organique Biologique, UMR 5623, Bât. IIR1, Université Paul Sabatier, 118 route de Narbonne, 31062 Toulouse Cedex, France

Abstract—Various dihydroxyacetone-phosphate (DHAP) analogues bearing an aromatic ring or β-dicarbonyl structures were synthesized. Their capacity to form a stabilized iminium ion or conjugated enamine in the reaction catalyzed by rabbit muscle aldolase (EC 4.1.2.13) were investigated by enzymatic kinetics and UV difference spectroscopic techniques. Whereas the aromatic derivative led to competitive inhibition without detectable iminium ion formation, slow reversible inhibitions of aldolase by β-dicarbonyl compounds was shown to have taken place. Conjugated enamine formation at the active site of the enzyme was detected by their specific absorbances close to 317 nm. Copyright © 1996 Elsevier Science Ltd

Introduction

The glycolytic enzyme fructose 1,6-diphosphate aldolase reversibly catalyzes the production of fructose 1,6-diphosphate (FDP) from D-glyceraldehyde 3-phosphate (GAP) and dihydroxyacetone phosphate.[1] There are two classes of aldolase:[2] class I aldolases proceed by Schiff base formation with the substrate, while class II require a metallic ion as a cofactor and do not form a Schiff base. Rabbit muscle aldolase is the most intensively studied class I aldolase and the aldol condensation proceeds by several ordered steps (Scheme 1): (i) iminium ion (or Schiff base) formation between the carbonyl of DHAP and the ε-amino group of an essential Lys residue (Lys 229);[3] (ii) enamine formation[4,5] after *pro*-S proton abstraction at C_3[6] in the iminium ion; (iii) enamine reaction with the carbonyl of GAP to form a new C–C bond and a second Schiff base; and (iv) hydrolysis of the latter iminium ion leading to FDP and free enzyme.



Scheme 1. Reaction catalyzed by aldolase and mechanistic pathway.

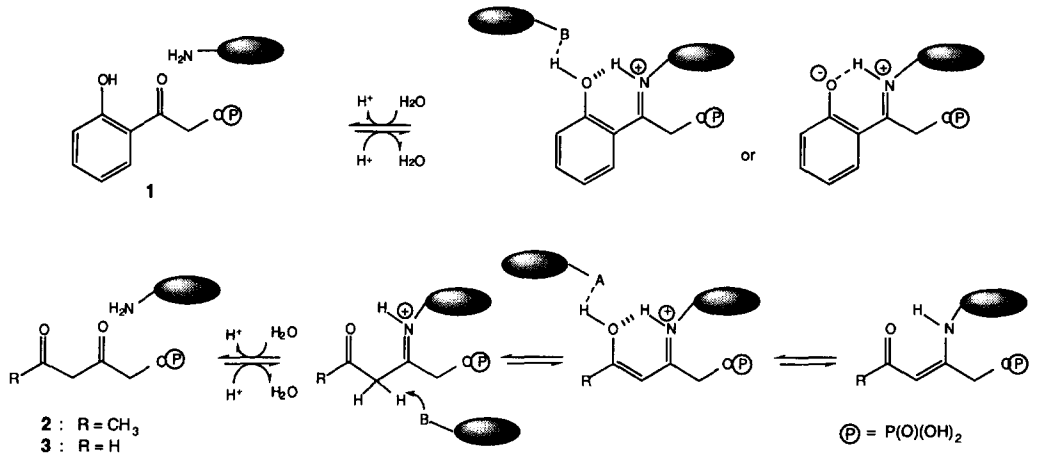
Working within the framework of a program concerned with glycolytic enzymes considered from a mechanistic point of view[7] and also as targets for the development of new antiparasitic drugs,[8] we became interested in the design of inhibitors. On the basis of the aldolase mechanistic pathway (Scheme 1), we considered the possibility of obtaining slow reversible inhibitions (slow-binding)[9] of this enzyme. This type of inhibition is generally associated with the stabilization of an intermediate reaction within the enzyme.[10] Only a few examples of slow-binding inhibitors are known for aldolase. One of them with D-erythrulose phosphate[11] and another with DHAP analogues modified at the C-3 locus.[7b] With these inhibitors, a Schiff base intermediate and possibly an enamine are members of the enzyme–inhibitor complex slowly formed and dissociated.

Compounds **1–3** potentially possess the ability to equally mimic such intermediates and different reactions could be expected (Scheme 2). Iminium ion formation for the three compounds and possible proton abstraction to lead to the conjugated enamine for β-dicarbonyl compounds **2** and **3**. The stabilization of these intermediates is possible through hydrogen bonding and structures in equilibrium. In order to investigate these possibilities, compounds **1–3** were therefore synthesized (Schemes 3 and 4) and assayed as aldolase inhibitors using enzymatic kinetics and UV difference spectroscopic techniques.

Results

Synthesis

Compounds **1–3** were synthesized according to Schemes 3 and 4. The phosphoric diester addition on



Scheme 2. Expected mechanistic pathway for aldolase inhibition by compounds **1–3**.

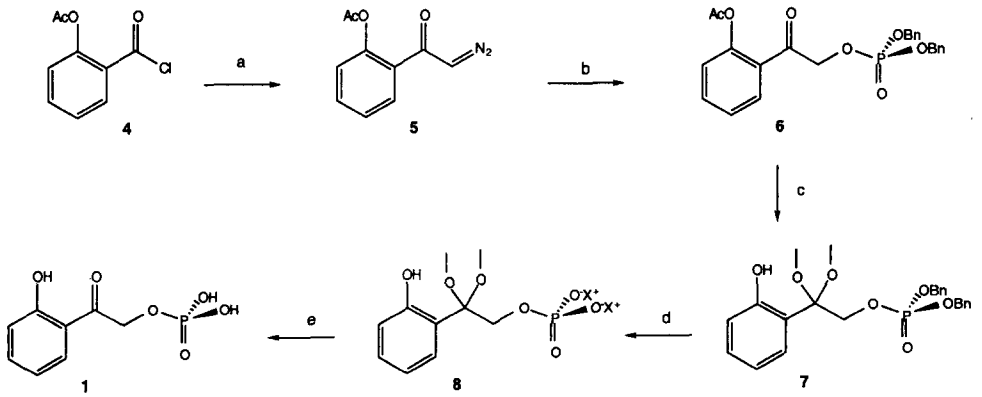
α-diazoketones is a straightforward route to introduce the β-keto phosphate moiety of DHAP in the synthesis of such compounds.[12,13] Indeed, the reaction was successfully used when the hydroxyl group (or phenol) of the corresponding diazoketone was protected by acetylation. With other protecting groups, such as benzyl, we mainly observed cyclization and rearrangement reactions.[14]

Compound **1** is easily obtained from 2-acetoxybenzoyl chloride **4** (Scheme 3), and compounds **2** and **3** from the β-propiolactone **9** and β-butyrolactone **10**, respectively (Scheme 4). The corresponding steps for **2** and **3** are as follows: the lactone (**9** or **10**) is opened by reaction of acetic acid[15] and the resulting product (**11** or **12**) transformed into the corresponding diazoketone (**13** or **14**) via the acyl chloride derivative. The reaction with dibenzylphosphate leads quantitatively to the phosphorylated compound (**15** or **16**). Hydroxyl group deprotection is performed by acidic methanolysis, conditions allowing the concomitant ketal formation of the carbonyl group acetal (compounds **17** or **18**). This protection is required to prevent proton abstraction and α-enone formation during the next oxidation step. In both cases, the oxidation is achieved with dimethylsulfoxide either in the presence of dicyclohexylcarbodiimide[16] (compound **19**), or pyridine–sulfur-trioxide complex[17] (compound **20**). The phosphate group is then deprotected by hydrogenolysis in the presence of palladium on charcoal, and compounds **21** or **22** are kept as cyclohexyl-ammonium salts. It is noteworthy that acetalization of compound **21** occurred concomitantly with the hydrogenolysis realized in anhydrous methanol.

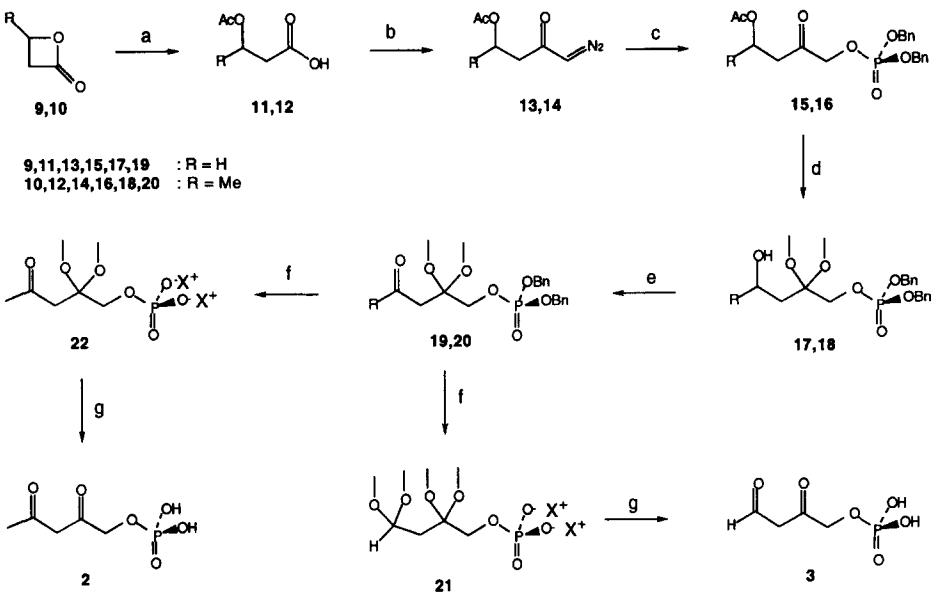
Before the enzymatic assays, compounds are deprotected by acidic hydrolysis of the ketal groups; ammonium to proton exchange on Dowex H^+ resins is followed by ketal hydrolysis catalyzed by the regenerated dihydrogenophosphate group. Finally, compounds **2** and **3** are obtained as aqueous solutions and characterized by their ^{13}C and ^{31}P spectra.

Enzymatic inhibition studies

Time effect on aldolase inhibition by compounds 1–3. Incubation of aldolase with compounds **1–3** leads to distinct situations (Table 1, enzyme concentration: 0.20 mg mL^{-1}). Whereas compound **1** induces no detectable enzymatic inhibition in the experimental conditions used, a time-dependent enzyme inhibition is observed with compounds **2** and **3**. Protection against



Scheme 3. Synthetic scheme for the synthesis of compound **1**. (a) CH_2N_2; (b) $(BnO)_2P(O)OH$; (c) $HC(OMe)_3$, MeOH, H^+; (d) (1) H_2, Pd/C (2) $C_6H_{11}NH_2$; (e) H_2O, Dowex H^+.



Scheme 4. Synthetic scheme for the synthesis of compounds **2** and **3**. (a) AcOH, H_2SO_4; (b) $(COCl)_2$; (c) $(BnO)_2P(O)OH$; (d) $HC(OMe)_3$, MeOH, H^+; (e) DMSO, C_5H_5N-SO_3, Et_3N, CH_2Cl_2 (17); DMSO, DCC, C_5H_5N, TFA, C_6H_6 (18); (f) (1) H_2, Pd/C (2) $C_6H_{11}NH_2$; (g) H_2O, Dowex H^+.

inhibition by DHAP suggests that inhibition is likely to occur at the active site. In contrast (not shown), no inhibition is detected with acetylacetone (10 mM), indicating the importance of both the phosphate group and the dicarbonyl structure of compounds **2** and **3**.

Competitive inhibition of aldolase by compound 1. Double reciprocal plots[18] (not shown) of the initial velocities of aldolase for different substrate and inhibitor **1** concentrations indicate that this compound is a competitive inhibitor with $K_i = 650$ μM.

Time-dependent reversible inhibition of aldolase by compound 2. Maximal residual activity of the enzyme at equilibrium reach 14% of the initial value for 3 mM of **2** (Table 2), showing that the enzyme is saturated in these conditions. The enzymatic activity is slowly but fully restored when an aliquot of the enzyme–inhibitor complex solution is diluted in the assay solution containing FDP at saturating concentration and auxiliary enzymes (measurements made in the FDP cleavage direction, Fig. 1). The time-dependent reversible inhibition observed, which exhibits saturation with increasing concentrations of the inhibitor, suggests that compound **2** behaves as a slow-binding inhibitor of aldolase (see equations in the Experimental).

Kinetic parameter values associated with this inhibition are summarized in Table 3. They are determined as follows: (1) first-order rate constant k_{-2}, from the data analysis for the restoration of the enzymatic activity (Fig. 1) using eq 1 that gives the product formation versus time for a slow-binding scheme; (2) dissociation constant K_i for the rapidly formed EI complex and the constant K corresponding to the equilibrium between EI and EI*, from data in Table 2 using eq 3 (not shown); and (3) first-order rate constant k_2 is obtained from K and k_{-2}. These data allow the determination of the overall inhibition constant K_i*.

Inhibition of aldolase by compound 3. Incubation of aldolase with compound **3** leads to a first-order loss of

Table 1. Time effect on aldolase inhibition by compounds **1–3**[a]

Compd	Concentration (mM)	Incubation time (min)	Remaining activity (%)
1	2	5	100
		15	100
	0.6	5	67 (95)[b]
		15	30 (94)[b]
2	3	5	24
		15	10
3	0.06	5	39 (82)[b]
		15	24 (75)[b]
	1	5	1

[a]Aldolase (0.20 mg mL^{-1}) was incubated in TEA buffer pH 7.6, at 25 °C with compounds **1–3** at the indicated concentrations. After 5 and 15 min incubation, the enzyme initial velocity was measured on 10 μL aliquots diluted in 1 mL solution assay so that the inhibitor concentration becomes negligible.
[b]The remaining activity when the experiment was run in presence of 1 mM DHAP is indicated between parentheses. A control experiment was carried out with the enzyme alone.

Table 2. Concentration effect of compound **2** on aldolase inhibition[a]

Compound **2** (mM)	Remaining activity (%)
0.3	38
0.6	27
1.2	19
3	14

[a]Aldolase was inhibited as shown in Table 1 by compound **2** at the indicated concentrations. For each inhibitor concentration, the maximum effect at equilibrium is indicated.

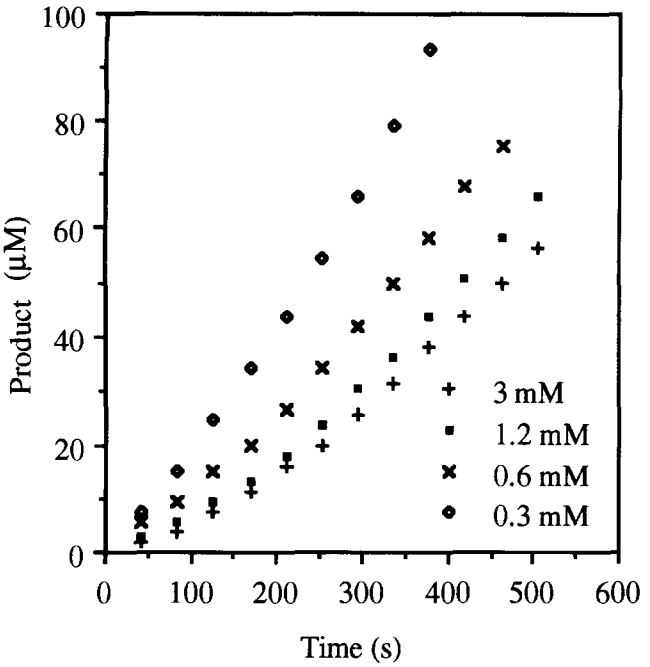


Figure 1. Reversion of aldolase inhibition by compound **2** in the presence of FDP. Aldolase was inhibited by compound **2** at the indicated concentrations as shown in Table 1. Reversion of inhibition was determinated on 10 μL aliquots diluted in 1 mL of assay solution containing 1 mM FDP (see Experimental). The rate value extrapolated to infinite time using eq 1 was found to be identical to that of the reference assay (made with the enzyme alone).

enzyme activity (Fig. 2). In the same conditions as those used for compound **2** (see Fig. 1), no significant restoration of enzyme activity is detected, allowing this compound to be considered as an irreversible inhibitor. Dissociation constant (K_i=900 μM) for the rapidly formed EI complex and first-order rate constant (k_2=0.70 min^{-1}) for the EI* complex formation are determined from the analysis of data in Figure 2 for an irreversible process (Fig. 3).

However, partial reversion of inhibition could be evidenced by adding samples of an aldolase solution previously inactivated by compound **3**, to a solution containing *p*-phenyl-diphosphate, a strong competitive inhibitor, in saturating concentration (Fig. 4, enzyme concentration: 10 mg mL^{-1}). The first-order rate constant ($k_{-2}=3.3\times10^{-3}$ min^{-1}) determined for this process is 200 times lower than the inactivation constant k_2, and shows that the determination of the latter by considering an irreversible inactivation can be accepted. Compound **3** therefore also behaves as a slow-binding inhibitor.

Table 3. Kinetic parameters for aldolase inhibitions by compound **2**

Constants	Values
k_{-2} (min^{-1})	0.074
K_i (μM)	1250
K	0.120
k_2 (min^{-1})	0.620
K_i* (μM)	130

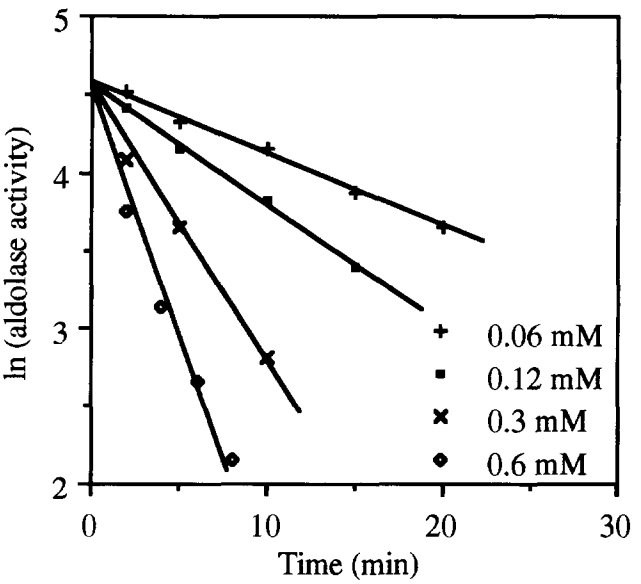


Figure 2. Inhibition of aldolase by compound **3**. Aldolase (0.20 mg mL^{-1}) was incubated in TEA buffer pH 7.60, 25 °C, with compound **3** at the indicated concentrations. At various times, residual activity was determined on 10 μL aliquots diluted in 1 mL of assay solution (see Experimental).

Schiff base formation between aldolase and compounds 1–3. An expected Schiff base formation was investigated by sodium borohydride treatment of enzyme–inhibitor complex solutions (Table 4, enzyme concentration: 1 mg mL^{-1}). After this reaction, the enzymatic inhibition initially generated by compound **3** becomes irreversible and no restoration of enzyme activity is obtained, even after addition of the strong competitive inhibitor *p*-phenyldiphosphate to the sodium borohydride treated enzyme–inhibitor complex solution. In contrast, a large part of this activity is slowly recovered after the same treatment in the case

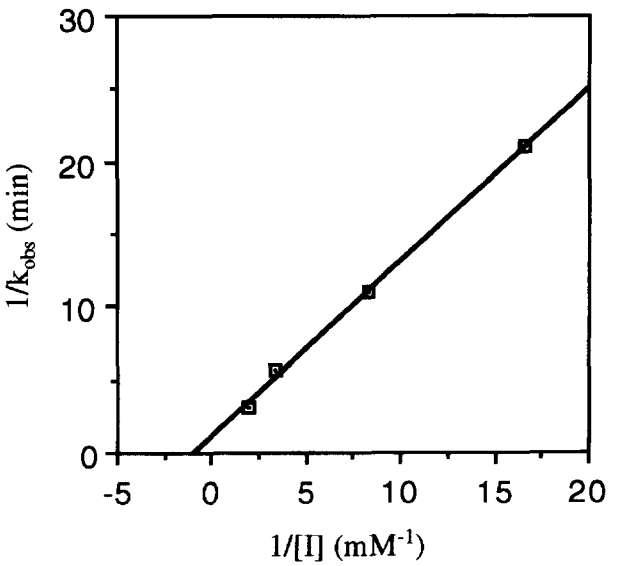


Figure 3. Compound **3** as irreversible aldolase inhibitor. Apparent first-order rate constant values k_{obs} for enzymatic inhibition were obtained from Figure 2.

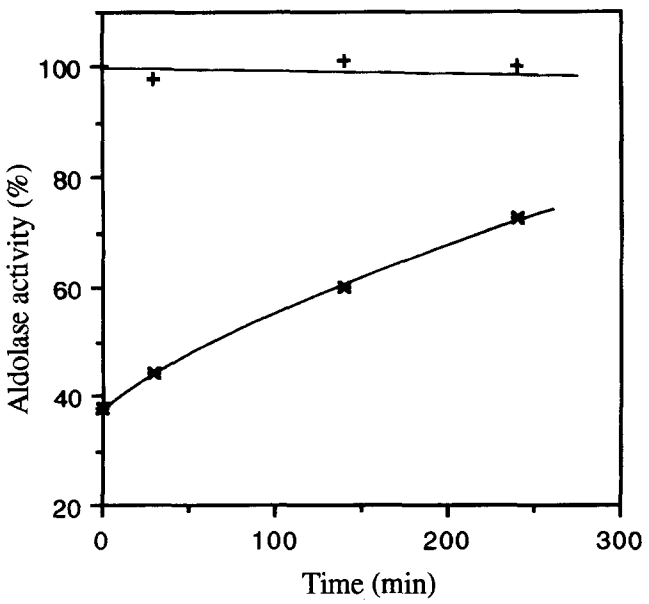


Figure 4. Partial reversion of aldolase inhibition by compound **3** in the presence of *p*-phenyldiphosphate. (*) aldolase inhibited by compound **3**; (+) control. Aldolase (10 mg mL^{-1}, TEA buffer pH 7.60, rt) was incubated with 3 mM compound **3** until 62% loss activity, then diluted 100-fold in TEA buffer containing *p*-phenyldiphosphate (5 mM) at 25 °C. At various times, residual activity was determined on 20 μL aliquots (see Experimental). The control was run without compound **3**. After a 24 h incubation with a competitive inhibitor, 80% of the initial activity was recovered.

of compound **2**, and no effect is evidenced with compound **1**. These results suggest that compound **3** forms an iminium ion to a larger extent than compound **2** and this intermediate is a part of the EI* complex. Conversely, this same possibility is ruled out for compound **1**, and the possible mechanistic pathway presented for this latter compound in Scheme 2 has to be discarded.

Table 4. Irreversible aldolase inhibition by sodium borohydride in the presence of compounds **1–3**

Compd	Concentration mM	Remaining activity (%)	
		[a]	[b]
DHAP	0.25	100	30
	2	100	20
1	1	95	95
	8	100	95
2	0.6	27	83[c]
3	0.1	35	35[c]

[a]Incubation without $NaBH_4$ treatment.
[b]Incubation with $NaBH_4$ treatment.
[c]20 min after $NaBH_4$ treatment, *p*-phenyldiphosphate (5 mM final concentration) was added. Assay solutions (0.2 mL of 0.1 M Tris buffer pH 6.0) contained aldolase (1 mg mL^{-1}), inhibitor or DHAP at concentrations indicated in the table. After 40 min incubation, reduction was performed by addition of 5 mL of a solution 0.25 M $NaBH_4$ in 1 mM sodium hydroxide; 150 min after sodium borohydride addition, 10 μL aliquots were withdrawn for measurement of the enzyme activity. In controls, where neither inhibitor nor DHAP, and with or without *p*-phenyldiphosphate had been added, only low inactivation (<5%) was observed after sodium borohydride addition.

UV difference spectroscopy studies

Interaction of compound 2 with aminocaproic acid. UV difference spectra were used for the reaction of compound **2** (50 μM) with aminocaproic acid (50 mM to 1 M) as a model for the interaction of this compound with the ε-amino group of Lys residues.[19] As shown in Figure 5A (given as an example), with increasing reaction time, through UV difference spectra, a first-order emergence of a differential absorbance maximal at 306 nm ($\Delta\varepsilon = 9000$ M^{-1} cm^{-1}) can be confirmed, ascribed to adduct formation. These changes reach a saturation plateau at high aminocaproic acid concentration. From the analysis of the differential absorbance changes at equilibrium versus aminocaproic acid concentrations, the dissociation constant ($K_d = 157$ mM) is determined for the adduct formed (Fig. 5B) using eq II-54 taken from Segel.[18]

Interaction of compound 2 with aldolase. The same type of UV difference spectra is obtained for the inter-

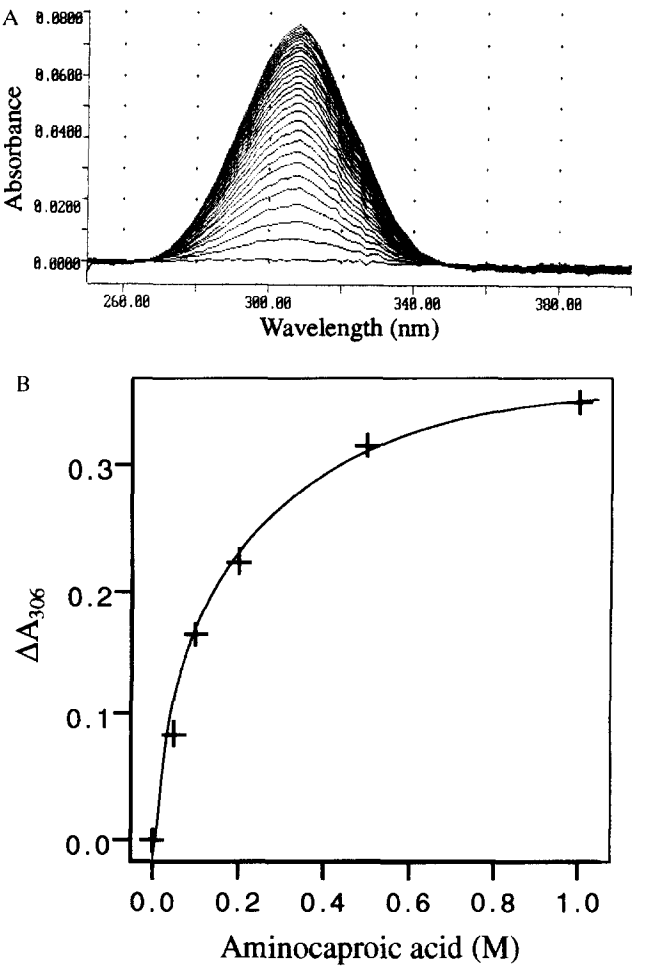


Figure 5. Interaction of aminocaproic acid with compound **2**. (A) UV difference spectra of aminocaproic acid + **2** versus aminocaproic acid; the concentration of **2** was 50 μM and in this example the concentration of aminocaproic acid was 50 mM and $\Delta t = 5$ min. (B) Change of A_{306} versus aminocaproic acid concentration. The points are experimental, and the line is the best fit to eq II-54 taken from Segel.[18]

action of aldolase (10 μM subunit) with compound **2** (50 μM to 1 mM); this leads to the emergence of a differential absorbance at 317 nm (Fig. 6A given as an example). However, this adduct formation can be analyzed in terms of two first-order kinetics: one fast and of high amplitude ($\Delta\varepsilon = 11400\ M^{-1}\ cm^{-1}$) and another 10-fold slower and threefold lower in absorbance change, which is not related to the inactivation (vide infra) and which has not been analysed further. The dissociation constant ($K_d = 150$ μM) for the enzyme–inhibitor complex is determined according to the same method as that used in Figure 5B (not shown). Observed first-order rate constants present saturation kinetics with increasing concentrations of compound **2** (Fig. 6B), a result consistent with a slow-binding inhibition scheme. The kinetic parameter values associated with the EI* complex formation are obtained in this second method, by fitting the data to eq. 2 (Fig. 6B and Table 5); the resulting values are in good agreement with those indicated in the first part (Table 3).

The reversion of the system is evidenced by adding FDP in saturating concentration (20 mM) to the EI* complex solution (Fig. 7). This reaction leads to a first-order decrease of the differential absorbance at 317 nm ($k_{-2} = 0.026\ min^{-1}$), value in agreement with that determined above (Table 5). Although the enzyme activity is fully restored after FDP addition, a fraction of the enzyme–compound **2** complex (about 30% of the initial value) is still present and therefore has no influence on this activity (Fig. 7). It is to be noted that this fraction is equivalent to that observed for the slow process of the biphasic enzyme–compound **2** complex formation.

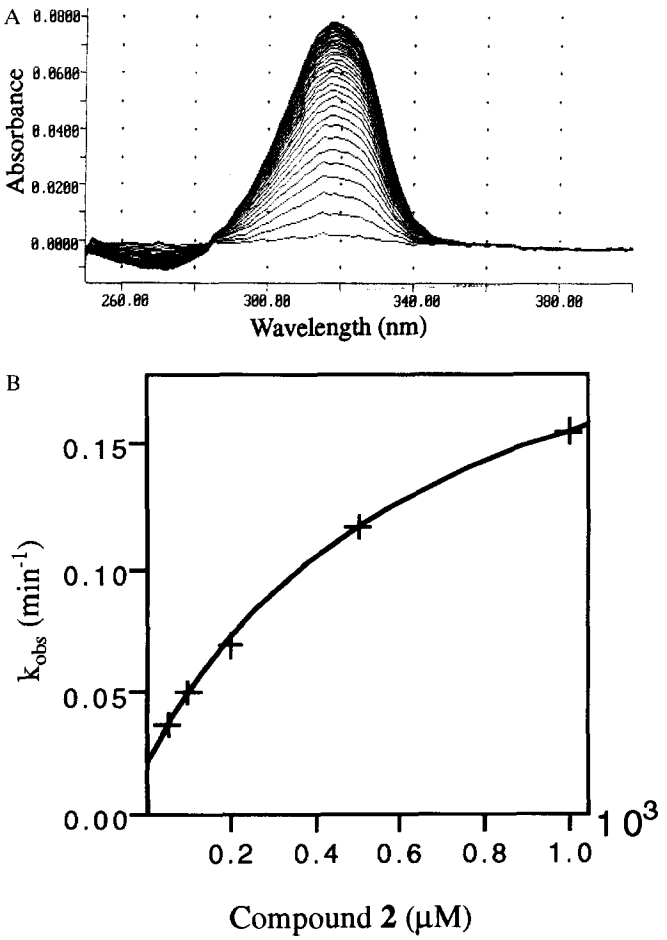


Figure 6. Interaction of compound **2** with aldolase. (A) UV difference spectra of 10 μM aldolase monomer + **2** versus **2**; in this example the concentration of **2** was 200 μM and $\Delta t = 2$ min. (B) Saturation kinetics of the enzyme–compound **2** complex formation determined at 317 nm. The points are experimental and the line is the best fit to eq 2 for a process made without substrate.

Table 5. Kinetic parameters for the Schiff base formation between aldolase and compound **2**

Constants	Values
k_{-2} (min^{-1})	0.0208
k_2 (min^{-1})	0.223
K_i (μM)	640
K_i^* (μM)	56

The nonspecific binding of compound **2** out of the active site of aldolase is supported by the fact that incubation of this compound with a β-glycerophosphate aldolase derivative[20] only gives the slow process (Table 6). The ε-amino group of the essential Lys residue is blocked in this modified aldolase obtained by sodium borohydride reduction of the DHAP–enzyme complex, where the remaining enzymatic activity corresponds to 1% of the initial value.[20]

Interaction of compound 3 with aminocaproic acid and aldolase. UV difference spectra for the interaction of

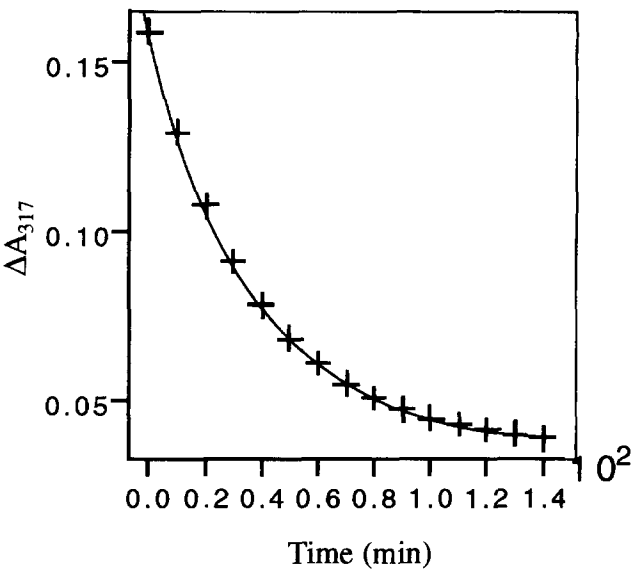


Figure 7. Interaction of FDP with aldolase–**2** complex. Replot of the change of A_{317} versus time. The points are experimental, and the line is the best fit to the first-order decay equation. The concentration of aldolase monomer was 10 μM and the concentration of **2** was 1 mM. At equilibrium, FDP (20 mM final concentration) was added and the change of A_{317} was determined versus time. The initial aldolase activity determined on aliquots in assay solution was recovered after this reaction.

Table 6. Enzyme–inhibitor complex formation between compound **2** and aldolase or β-glycerophosphate aldolase[a]

Aldolase	k_{obs} (min^{-1})	ΔA (317 nm)
Native	0.179	0.1065
	0.025	0.035
β-Glycerophosphate	0.021	0.038

[a]UV difference spectroscopy kinetic parameters for enzyme–inhibitor complex formation between aldolase or β-glycerophosphate aldolase (10 μM subunit each, TEA buffer pH 7.60, 25 °C) and compound **2** (1 mM) were determined at 317 nm.

compound **3** (50 μM) with aminocaproic acid (0.1–100 mM) demonstrate a first-order emergence of two differential absorbances, one with a maximum at 317 nm ($\Delta\varepsilon = 9600\ M^{-1}\ cm^{-1}$) due to the adduct formation, and the other with a minimum at 283 nm ($\Delta\varepsilon = -4600\ M^{-1}\ cm^{-1}$) due to the decrease of the aldehyde and with an isosbestic point at 298 nm (not shown). The dissociation constant (K_d) for the adduct formed is found close to 0.5 mM. Comparatively, the differential maximum and minimum absorbances are shifted to 325 and 292 nm, respectively, in the UV difference spectra for the interaction of aldolase (10 μM subunit) and compound **3** (60 and 100 μM, data not shown). The absorbance changes are consistent with the sum of two first-order kinetics, as for aldolase and compound **2** (Table 6), and the use of β-glycerophosphate aldolase derivative suppresses the faster kinetics (data not shown), suggesting that this latter is associated with a reaction involving the active site of aldolase. Moreover, as the reaction time increases, the isosbestic point is disrupted indicating the presence of intermediates and side reactions. These latter could account both for the overall enzymatic inhibition induced by this compound and for the incomplete restoration of enzyme activity in the presence of a strong competitive inhibitor (Fig. 4). This proposal is supported by the strongly lowered but still present inhibition of aldolase by this compound in the presence of saturating concentration of DHAP (Table 1). For compound **2**, the protection against inhibition by DHAP is more effective than with **3**.

Discussion

Previous results from the literature suggest that the slowly reversible inhibition by DHAP analogues can be obtained for fructose 1,6-biphosphate aldolase.[7b,11] Such a situation can be accounted for by stabilization of one of the reaction intermediates formed along the reaction pathway of aldolase, iminium ion or enamine, analogous to those formed with the normal substrate DHAP or FDP.

The reaction of the ε-amino group of essential Lys 229 residue of aldolase with compounds **1–3** (Scheme 1) has been considered in this work, with covalent formation of either a stable Schiff base complex, for compound **1**, or a stabilized enamine, in fact a vinylogous amide, for compounds **2** and **3**. For compound **1**, only a weak, time-independent enzymatic inhibition is observed. Attempts to prove the fast equilibrium of a Schiff base formation by sodium borohydride trapping failed. It turns out that compound **1** behaves as a competitive inhibitor with an inhibition constant of $K_i = 650$ μM. This value is to be compared to that of other monophosphorylated derivatives not bearing an aromatic ring such as alkyl monoglycolate phosphoric esters[21] whose inhibition constants (K_i values) are in the range of 10 mM; this suggests that the aromatic ring in compound **1** contributes to affinity with aldolase. The absence of a detectable Schiff base formation could be due to steric hindrance in this structure.[7b]

In contrast, a slowly reversible inhibition is observed with compounds **2** and **3**, and the β-dicarbonyl structure of these compounds is required for any inhibition to be observed. The importance of such a β-dicarbonyl structure has previously been mentioned in the case of another enzyme giving a Schiff base intermediate, the aceto-acetate decarboxylase enzyme which is inhibited by acetyl-acetone[22] or aceto-pyruvate,[23] also through a slowly reversible process. In these latter cases, the inhibition process is associated with the formation of a conjugated enamine, similar to that shown in Scheme 1. Concerning aldolase, inhibition by the dicarbonyl compounds **2** and **3** requires the presence of the phosphate group on the inhibitor (acetyl-acetone has no effect on the enzyme, similarly to the fact that dihydroxyacetone is not a substrate for this enzyme[24]). This phosphate group likely binds at Arg 148 as the phosphate group in DHAP does.[3c]

The characterization of the conjugated enamine formation between an ε-amino group of lysine residue in aldolase and compounds **2** and **3** was obtained by UV difference spectroscopy. The difference spectra exhibit an absorbance with a maximum close to 317 nm, specific for such intermediates.[23,25] However, results obtained with compounds **2** and **3** present distinct patterns. With the diketo compound, the study of the aldolase–inhibitor interaction can be achieved using either inhibition kinetics (and that of activity restoration) or kinetics followed by UV difference spectroscopy of the formation and disappearance of the enzyme–inhibitor complex. The kinetic parameters obtained by the two methods are in good agreement (Tables 3 and 5), particularly when the completely different approaches are considered. Incubation of aldolase with different concentrations of compound **2** leads to inhibition plateaus (Table 2) with concomitant appearance of an absorbance with a maximum at 317 nm (Fig. 6A), indicating that the two processes are associated. The spectroscopic data show that the conjugated enamine formation is approximately one thousand times easier with aldolase ($K_d = 150$ μM) than with aminocaproic acid ($K_d = 170$ mM), the compound used as model of the ε-amino group of lysil residue (Fig. 5).

The restoration of the enzymatic activity obtained by displacement of inhibitor **2** from the EI* complex by

FDP addition (Figs 1 and 6B) indicates the reversibility of the process; however, it should be noted that although the reversion is complete in these conditions, a part of the enzyme–inhibitor complex is conserved, in the range of 30% of the initial value. This means that the inhibitor also binds out of the active site, and that this nonspecific binding has no detectable effect on the activity of the recovered enzyme. This 30% amount corresponds to that determined for the slow kinetics of the biphasic process of aldolase–inhibitor complex formation, when followed by the spectroscopic method (Table 6). As previously reported for similar situations,[22,23] the conjugated enamine formed between aldolase and compound **2** is not quantitatively reduced by sodium borohydride (Table 4). To confirm that the enamine formed with compound **2** during, first, the inactivation process, and second, that of DHAP during FDP synthesis, involves the same ε-amino group of Lys residue, the reaction of compound **2** was investigated with β-glycerophosphate aldolase.[20] With this modified enzyme, blocked at the Lys 229 site, only the part corresponding to the aldolase–compound **2** complex, not related to the enzyme inactivation, was observed (Table 6).

The interaction of compound **3** with aldolase was also studied using the same two methods. The enzyme inhibition process is first-order and the corresponding rate constant is in the same range as that obtained with compound **2** (Table 3). This reaction is also characterized in UV difference spectroscopy by the absorbance at 325 nm. Again, the complex formation corresponds to two first-order processes, the faster one being suppressed when the experiment is made with the inactivated β-glycerophosphate aldolase. The essential ε-amino group of Lys 229 residue is therefore also involved in the inhibition by compound **3**. The partial reversion of inhibition in the presence of a competitive inhibitor (Fig. 4) is suppressed when the enzyme–inhibitor complex is pretreated with a solution of sodium borohydride (Table 4). But owing to the likely presence of a reactive aldehyde group, **3** is prone to react with other residues, in a nonspecific binding as do several different aldehydes with aldolase in the absence of DHAP.[26,27] Another feature may account for the difference observed between compounds **2** and **3**: the higher acidity of the methylene inserted between the two carbonyl groups in the aldehyde (p*K* 5.9 for the acetylpropanal) compared to **2** (p*K* 9 for acetylacetone). Aldehyde **3** may therefore react in the enolate form, able to abstract a proton to different lysil groups, therefore giving less selectivity than compound **2** towards Lys 229. This higher reactivity of **3** versus **2** is also observable in the reaction with aminocaproic acid, the corresponding complexes having dissociation constants of 0.5 and 170 mM, respectively.

Although they present some differences in their behaviour towards aldolase, compounds **2** and **3** are clearly slow-binding inhibitors for this enzyme, a property related to their ability to produce stabilized intermediates similar in structure to those formed in the normal process. Interestingly, it has recently been shown that the same β-dicarbonyl structure is essential in haptens that have been used to produce antibodies, which reveal a class I aldolase activity.[25] Noteworthy is also the fact that the antibody forms a complex absorbing at 317 nm with the hapten, a wavelength close to that corresponding to the complex formed between inhibitor **2** or **3** and aldolase. On the grounds of this parallel, the second carbonyl group, ketone for compound **2**, aldehyde for compound **3**, can be considered as a mimic of the incoming (or leaving) second reactant (GAP) of the aldolase catalyzed reaction.

Experimental

Enzymes and reagents

FDP sodium salt, DHAP lithium salt, glycerol phosphate dehydrogenase, triose-phosphate isomerase, and rabbit muscle aldolase were purchased from Boehringer Mannheim. All other chemicals were purchased from Aldrich and were used without extra purification. β-Glycerophosphate aldolase derivative was obtained by sodium borohydride treatment of a solution of aldolase and DHAP as previously described.[20] The enzymatic activity of β-glycerophosphate aldolase was 1% of the initial value. *p*-Phenyl diphosphate, competitive inhibitor of aldolase ($K_i = 40$ μM), was synthesized according to the protocol described previously.[28]

Assay methods

Aldolase activity (10 units mg^{-1} at 25 °C) was measured by means of triose-phosphate isomerase/glycerol-1-phosphate dehydrogenase method in 1 mL of 0.1 M triethanolamine hydrochloride buffer (1 mM EDTA, pH 7.6 and ionic strength 0.15), using FDP (1 mM) as substrate as described elsewhere.[7b,29] Aldolase was dialyzed overnight at 4 °C against TEA buffer before use and the protein concentration was estimated using $A^{1\%}$ (280 nm) = 9.1.[30] The subunit concentration was determined assuming a molecular weight of 159000 for the tetrameric aldolase.[31]

Kinetic parameters and equilibrium constants values determination

The data were subjected to a nonlinear least square fit to the appropriate equation using the Ultrafit program.[32]

Inhibition study

Aldolase (0.2 mg mL^{-1} in 0.2 mL TEA buffer) was incubated in the presence of the compound under study (0.06–3 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.

Competitive inhibition study

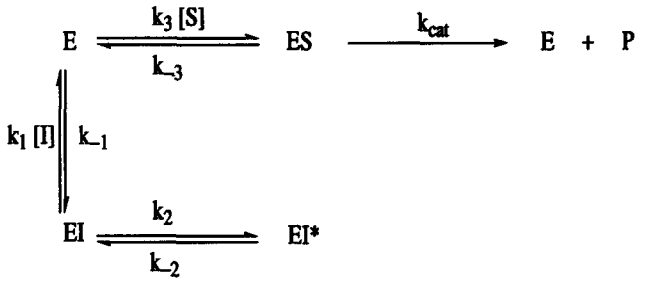
The dissociation constant of the enzyme–inhibitor complex was determined by double reciprocal plots of initial velocities of aldolase (2 μg mL^{-1}) for FDP and compound **1** concentrations in the range of 10–100 μM and 0.6–1.2 mM, respectively.

Sodium borohydride reductions

Reductions of the aldolase–inhibitor complexes were performed according to a previously described technique.[33]

Equations

For the general system:



P production versus time is given by the following equation:

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

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.[9]

For this system:

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

where K_i, 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. The value of the apparent first-order rate constant (k') increases hyperbolically as a function of I with lower and upper limits k_{-2} and $k_{-2}+k_2$, 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:

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

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

$$\frac{[E_t]}{[EI^*]} - 1 = K + \frac{K K_i}{[I]} \quad (3) \qquad K_i^* = \frac{K_i k_{-2}}{k_2 + k_{-2}},$$

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

UV difference spectroscopy

Absorbance spectra were measured using a Cary 1E Varian spectrophotometer at constant 25 °C. The same TEA buffer as for enzymatic kinetic studies was utilized. Absorbance spectra were measured using a previously described method.[19] Absorption spectra were either scanned between 250 and 400 nm or at wavelengths corresponding to maximum (or minimum) absorption and recorded as a function of time. Measurements were initiated by addition of the compound under study at various final concentrations to TEA buffer solutions containing a fixed concentration of aldolase (10 μM subunit). The measured absorption spectra of the enzyme complex recorded at timed intervals was corrected for absorption by buffer, compound under study and the enzyme alone. For the model study, in each assay, aminocaproic acid was added at different final concentrations to TEA buffer solutions containing a fixed concentration of compounds **2** or **3** (50 μM). Absorption spectra were recorded at timed intervals and corrected for absorption by buffer, compounds **2** or **3** and aminocaproic acid. Observed apparent first-order rate constants, k_{obs}, and limiting maximal absorption differences, ΔA_{max}, were obtained for each assay by fitting the time-dependent absorption data against a first-order kinetic equation (or the sum of two first-order kinetic processes) using the Ultrafit program. The dissociation constant K_d was extracted from the determined maximal absorption differences according to equation II-54 taken from Segel.[18] The dissociation constant K_i for the rapidly formed complex aldolase–compound **2** or **3** and rate constants k_2 and k_{-2} corresponding, respectively, to the formation and dissociation of the slow reacting aldolase–inhibitor complex were derived from analysis of observed apparent rate constants as shown in eq 2. In the case of compound **2**, the first-order rate constant k_{-2} was also derived independently from absorbance data corresponding to displacement of this inhibitor from the EI* complex by 20 mM FDP.

Synthesis

NMR spectra were recorded in $CDCl_3$ or D_2O on a Bruker AC80 spectrometer. All chemical shifts (δ) are reported in parts per million (ppm) with respect to TMS for ^1H and ^{13}C spectra, H_3PO_4 for ^{31}P spectra as an internal standard.

***ortho*-Acetoxy-ω-diazoaketophenone (5).** To a soln of CH_2N_2 (47 mmol) in 100 mL Et_2O at −70 °C (prepared according to the manufacturer's protocol) a solution of 2-acetoxybenzoyl chloride **4** (1.99 g, 10

mmol) in 10 mL Et_2O was added dropwise. After addition, the reaction mixture was stirred at rt for 2 h. Excess CH_2N_2 was removed by purging with Ar and destroyed in an HOAc solution. The solvent was removed by evapn in vacuo and the remaining residue provided, after recrystallization in hexane, diazoketone **5** as a slightly yellow powder (1.52 g, 74%). IR (KBr) 3079, 2102, 1750, 1600 cm^{-1}. ^1H NMR ($CDCl_3$, 80 MHz): δ 2.32 (s, 3H), 5.72 (s, 1H), 7.37 (m, 4H). Mass spectrometry (DCI/NH_3) *m/z* 222 $[M+NH_4]^+$, 205 $[M+H]^+$.

[2-(*ortho*-Acetoxy-phenyl)-2-oxo]-ethyl dibenzylphosphate (6). A mixture of diazoketone **5** (1 g, 4.9 mmol) and dibenzylphosphate (2.03 g, 7.3 mmol) in 50 mL of dry benzene was stirred at 80 °C for 7 h. Et_2O (50 mL) was added and the resulting mixture was washed with a satd soln of sodium bicarbonate (50 mL) then water (50 mL).The organic layer was dried over $MgSO_4$ and evapd in vacuo to yield **6** as a yellow oil (2 g, 90%), which was used in the next step without further purification. IR (neat) 1769, 1712 cm^{-1}. ^1H NMR ($CDCl_3$, 80 MHz): δ 2.29 (s, 3H), 5.05 (d, $^3J_{HP}=10.1$ Hz, 2H), 5.11 (d, $^3J_{HP}=8.0$ Hz, 4H), 7.8–6.8 (m, 14H). ^{31}P NMR ($CDCl_3$, 81 MHz): δ −0.89. Mass spectrometry (DCI/NH_3) *m/z* 472 $[M+NH_4]^+$, 455 $[M+H]^+$.

[2-(*ortho*-Hydroxy-phenyl)-2,2-dimethoxy]-ethyl dibenzylphosphate (7). A mixture of compound **6** (1 g, 2.2 mmol), methyl orthoformate (2.33 g, 22 mmol), and concd sulfuric acid (59 μL, 1.1 mmol) in 10 mL MeOH was stirred at rt for 24 h. Et_2O (50 mL) was added, and the resulting mixture washed with a satd soln of sodium bicarbonate (25 mL), water (25 mL), and brine (25 mL). The organic layer was dried over $MgSO_4$ and evapd in vacuo. The remaining product was purified by flash chromotography (toluene:EtOAc, 8:2) to yield **7** as a white powder (0.700 g, 69%). IR (KBr) 3270, 1278, 1033 cm^{-1}. ^1H NMR ($CDCl_3$, 200 MHz): δ 3.30 (s, 6H), 4.17 (d, $^3J_{HP}=5.6$ Hz, 2H), 4.81 (d, $^3J_{HP}=8.0$ Hz, 4H), 7.5–6.8 (m, 14H), 8.7 (s, 1H, OH). ^{13}C NMR ($CDCl_3$, 50 MHz): δ 49.22, 67.67 (d, $^2J_{CP}=5.4$ Hz), 69.31 (d, $^2J_{CP}=5.7$ Hz), 104.42 (d, $^3J_{CP}=10$ Hz), 117.58, 118.73, 120.53, 128.31, 130.56, 128, 128.5, 135.8, 157.08. ^{31}P NMR ($CDCl_3$, 81 MHz): δ −1.67. Mass spectrometry (DCI/CH_4) *m/z* 467 $[M+C_3H_5-MeOH]^+$, 459 $[M+H]^+$, 455 $[M+C_2H_5-MeOH]^+$, 427 $[M+H-MeOH]^+$, 395 $[M+H-2MeOH]^+$. Calcd for $C_{24}H_{27}O_7P$: C, 62.88; H, 5.94. Found: C, 62.84; H, 5.96.

[2-(*ortho*-Hydroxy-phenyl)-2,2-dimethoxy]-ethyl phosphate bis-cyclohexylammonium salt (8). A mixture of protected phosphate **7** (0.300 g, 0.65 mmol) and 10% Pd on charcoal (100 mg) in 10 mL MeOH was hydrogenated over 30 min. Freshly distilled cyclohexylamine (0.130 g, 1.3 mmol) in 5 mL MeOH was added. The catalyst was eliminated by filtration and copiously washed with MeOH. The solvent was evaporated in vacuo, the remaining product was dissolved in water (20 mL) and the resulting solution washed with hexane (20 mL). The aq layer was lyophilized to yield **8** as a white powder (0.265 g, 86%). ^1H NMR (CD_3OD, 200 MHz): δ 2–1 (m, 20H), 2.9 (m, 2H), 3.34 (s, 6H), 4.09 (d, $^3J_{HP}=3.5$ Hz, 2H), 7.3–6.7 (m, 4H). ^{13}C NMR (CD_3OD, 50 MHz): δ 25.57, 26.12, 32.79, 49.89, 51.25, 67.46, 106.14, 117.97, 120.44, 120.69, 130.12, 130.99, 157.86. ^{31}P NMR (CD_3OD, 81 MHz): δ 3.96.

[2-(*ortho*-Hydroxy-phenyl)-2-oxo]-ethyl dihydrogenophosphate (1). To a soln of compound **8** (0.100 g, 0.21 mmol) in 5 mL water, resin Dowex 50WX8 (H^+ form, 4 mL) was added and the mixture was stirred smoothly for 1 h. The resin was eliminated by filtration and washed by water (10 mL). The combined aq solns were adjusted to pH 6.5 with KOH (0.1 M), washed twice with Et_2O (20 mL) and lyophilized to yield **1** monopotassium salt as a white powder (0.050 g, 90%). ^1H NMR (D_2O, 200 MHz): δ 5.21 (d, $^3J_{HP}=7.2$ Hz, 2H), 7.63–7.0 (m, 4H). ^{13}C NMR (D_2O, 50 MHz): δ 69.47 (d, $^2J_{CP}=3.8$ Hz), 120.28, 120.39, 122.72, 132.13, 139.75, 162.53, 204.51 (d, $^3J_{CP}=7$ Hz). ^{31}P NMR (D_2O, 81 MHz): δ 2.15.

Compounds **2** and **3** were synthesized in the same way. The synthesis of compound **3** is given as an example.

3-Acetoxy-propanoic acid (11). A mixture of β-propiolactone **9** (3.44 g, 4.77 mmol) and concd sulfuric acid (5 μL, 90 μmol) in 14 mL HOAc was stirred for 2 h at room temperature.[15] The solvent was removed by evapn in vacuo and the remaining product after flash distillation (0.1 torr, 115 °C) yielded compound **11** as a colorless oil (3.79 g, 60%). IR (neat) 3600–2500, 1760, 1720 cm^{-1}. ^1H NMR ($CDCl_3$, 80 MHz): δ 1.99 (s, 3H), 2.64 (t, $^3J=6.4$ Hz, 2H), 4.28 (t, $^3J=6.4$ Hz, 2H), 10.28 (s, 1H, OH).

1-Diazo-4-acetoxy-2-butanone (13). To a soln of acid **11** (1.32 g, 10 mmol) in 15 mL of dry CH_2Cl_2, oxalylchloride (2.54 g, 20 mmol) in 10 mL of dry CH_2Cl_2 was added dropwise and the mixture stirred for 2 h at 40 °C. Excess oxalylchloride and most of the solvent were removed by distillation. The remaining soln, diluted in 5 mL of dry CH_2Cl_2, was added dropwise at −70 °C to a soln of CH_2N_2 (46 mmol) in Et_2O (100 mL). The reaction mixture was stirred for 1 h at rt. Excess CH_2N_2 was removed by purging with Ar and destroyed in an HOAc solution, the solvent was removed by evaporation in vacuo to yield diazoketone **13** as a yellow oil (1.50 g, 96%), which was used rapidly in the next step. IR (neat) 2108, 1736, 1638 cm^{-1}. ^1H NMR ($CDCl_3$, 80 MHz): δ 2.02 (s, 3H), 2.63 (t, $^3J=6.3$ Hz, 2H), 4.34 (t, $^3J=6.3$ Hz, 2H), 5.26 (s, 1H).

(4-Acetoxy-2-oxo)-butyl dibenzylphosphate (15). A mixture of diazoketone **13** (1.50 g, 96 mmol) and dibenzylphosphate (4.17 g, 15 mmol) in 15 mL benzene was stirred for 3 h at 70 °C. The solvent was evapd in vacuo and the remaining residue dissolved in 50 mL Et_2O. The solution was washed with a satd soln of sodium bicarbonate (50 mL), with water (50 mL) then with brine (50 mL), dried over $MgSO_4$ and evapd in vacuo to yield compound **15** (4 g, 90%), which was used in the next step without further purification. IR (neat) 1736 cm^{-1}. ^1H NMR ($CDCl_3$, 80 MHz): δ 1.98 (s, 3H),

2.66 (t, $^3J=6.0$ Hz, 2H), 4.26 (t, $^3J=6.0$ Hz, 2H), 4.44 (d, $^3J_{HP}=9.0$ Hz, 2H), 5.08 (d, $^3J_{HP}=8.7$ Hz, 4H), 7.33 (s, 10H). ^{13}C NMR ($CDCl_3$, 50 MHz): δ 20.8, 37.5, 58.5, 69.87 (d, $^2J_{CP}=5.6$ Hz), 70.55 (d, $^2J_{CP}=5.9$ Hz), 128.1, 128.5, 135.5, 202.0 (d, $^3J_{CP}=6.6$ Hz). ^{31}P NMR ($CDCl_3$, 81 MHz): δ −1.07.

(4-Hydroxy-2,2-dimethoxy)-butyl dibenzylphosphate (17). A mixture of compound **15** (1 g, 2.46 mmol), methyl orthoformate (0.262 g, 2.47 mmol) and concd sulfuric acid (90 μL, 1.6 mmol) in 3 mL MeOH was stirred for 16 h at rt, Et_2O (20 mL) was added and the mixture washed by a satd soln of sodium bicarbonate (20 mL), water (20 mL), and brine (20 mL). The organic layer was dried over $MgSO_4$ and the solvent evapd in vacuo. The crude residue was purified by flash chromatography (CH_2Cl_2:MeOH, 97:3) to yield **17** as a slightly yellow oil (0.710 g, 70%). IR (neat) 3420, 1267, 1034 cm^{-1}. ^1H NMR ($CDCl_3$, 200 MHz): δ 1.96 (t, $^3J=6.1$ Hz, 2H), 2.20 (s, 1H, OH), 3.19 (s, 6H), 3.65 (t, $^3J=6.1$ Hz, 2H), 4.00 (d, $^3J_{HP}=6.5$ Hz, 2H), 5.04 (d, $^3J_{HP}=8.35$ Hz, 4H), 7.32 (s, 10H). ^{13}C NMR ($CDCl_3$, 50 MHz): δ 34.23, 48.33, 58.28, 65.35 (d, $^2J_{CP}=5.6$ Hz), 69.58 (d, $^2J_{CP}=5.5$ Hz), 100.66 (d, $^3J_{CP}=8.9$ Hz), 128.0, 128.80, 135.70. ^{31}P NMR ($CDCl_3$, 81 MHz): δ −0.74. Anal. calcd for $C_{20}H_{27}O_7P$: C, 58.53; H, 6.63; O, 27.29; P, 7.54. Found: C, 57.79; H, 6.76; O, 27.68; P, 7.77.

(2,2-Dimethoxy-4-oxo)-butyl dibenzylphosphate (19). A mixture of compound **17** (0.400 g, 0.97 mmol), dimethylsulfoxide (1.9 mL), triethylamine (0.675 mL, 4.85 mmol), sulfur-trioxide–pyridine17 (0.77 g, 4.85 mmol) and CH_2Cl_2 (1.5 mL) was stirred at rt for 1 h, Et_2O (20 mL) was added and the mixture washed twice with water (20 mL). The organic layer was dried over $MgSO_4$ and the solvent evapd in vacuo. The remaining residue was purified by flash chromatography (toluene:EtOAc, 6:4) to yield **19** as a slightly yellow oil (0.360 g, 91%). IR (neat) 1722 cm^{-1}. ^1H NMR ($CDCl_3$, 200 MHz): δ 2.66 (d, $^3J=2.8$ Hz, 2H), 3.20 (s, 6H), 4.00 (d, $^3J_{HP}=5.7$ Hz, 2H), 5.03 (d, $^3J_{HP}=7.9$ Hz, 4H), 7.34 (s, 10H), 9.62 (t, $^3J=2.8$ Hz, 1H, HCO). ^{13}C NMR ($CDCl_3$, 50 MHz): δ 46.80, 48.20, 65.77 (d, $^2J_{CP}=5.5$ Hz), 69.64 (d, $^2J_{CP}=5.6$ Hz), 99.20 (d, $^3J_{CP}=10.4$ Hz), 128.00, 128.70, 135.60, 199.40. ^{31}P NMR ($CDCl_3$, 81 MHz): δ −1.09. Mass spectrometry (DCI/NH_3) *m/z* 409 $[M+H]^+$, 426 $[M+NH_4]^+$, 377 $[M+H-MeOH]^+$. Anal. calcd for $C_{20}H_{25}O_7P$: C, 58.82; H, 6.17. Found: C, 58.60: H, 6.26.

(2,2,4,4-Tetramethoxy)-butyl phosphate, bis-cyclohexylamonium salt (21). Following the procedure for the synthesis of compound **8**, compound **19** (0.100 g, 0.24 mmol) yielded **21** bis-cyclohexylamonium salt as a white powder (0.095 g, 86%). ^1H NMR (CD_3OD, 200 MHz): δ 2.0–1.0 (m, 20H), 2.05 (d, $^3J=5.0$ Hz, 2H), 3.0 (m, 2H), 3.24 (s, 6H), 3.34 (s, 6H), 3.83 (d, $^3J_{HP}=3.75$ Hz, 2H), 4.60 (t, $^3J=5.0$ Hz, 1H). ^{13}C NMR (CD_3OD, 50 MHz): δ 25.59, 26.11, 32.40, 36.81, 48.59, 51.18, 53.70, 63.75 (d, $^2J_{CP}=4.5$ Hz), 101.87 (d, $^3J_{CP}=11.3$ Hz), 103.34. ^{31}P NMR (CD_3OD, 81 MHz): δ 3.84. Anal. calcd for $C_{20}H_{45}N_2O_8P$, $2H_2O$: C, 47.23; H, 9.71; N, 5.51. Found: C, 47.05; H, 9.55; N, 5.58.

(2,4-Dioxo)-butyl phosphate (3). To a soln of compound **21** (0.090 g, 0.19 mmol) in water (0.5 mL), resin Dowex 50WX8 (H^+ form, 0.5 mL) was added. The mixture was stirred smoothly for 5 min and the resin was separated by filtration and washed by water (0.5 mL). The combined aq solns were incubated for 24 h at rt and neutralized (pH 7.60) with KOH (0.10 M). Compound **3** soln was titrated with KOH (0.01 M). ^{13}C NMR (D_2O+H_2O, 50 MHz): δ 49.00, 72.66 (d, $^2J_{CP}=4.5$ Hz), 89.93 ($HC(OH)_2$), 208.7. ^{31}P NMR (D_2O+H_2O, 81 MHz): δ 0.40.

References

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

2. Rutter, W. J. *Fed. Proc.* **1964**, *23*, 1248.

3. (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; (c) Sygusch, J.; Beaudry, D.; Allaire, M. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 7846.

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

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

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

7. (a) Gefflaut, T.; Blonski, C.; Périé, J.; Willson, M. *Prog. Biophys. Molec. Biol.* **1995**, *63*, 301; (b) Blonski, C.; Gefflaut, T.; Périé, *J. Bioorg. Med. Chem.* **1995**, *3*, 1247.

8. (a) Périé, J.; Riviere-Alric, I.; Blonski, C.; Gefflaut, T.; Lauth de Viguerie, N.; Trinquier, M.; Willson, M.; Opperdoes, F.; Callens, M. *Pharmac. Ther.* **1994**, *60*, 347; (b) Willson, M.; Callens, M.; Kuntz, D.; Périé, J.; Opperdoes, F. *Molec. Biochem. Parasitol.* **1993**, *59*, 201; (c) Willson, M.; Lauth de Viguerie, N.; Périé, J.; Opperdoes, F.; Callens, M. *Biochemistry* **1994**, *33*, 214.

9. (a) Willams, J. W. W.; Morrison, J. F. *Meth. Enzymol.* **1979**, *63*, 437; (b) Morrison, J. F.; Walsh, C. T. In *Advanced Enzymology*; Meister, A., Ed.; Interscience: New York, 1988; Vol. 61, p 201.

10. Schloss, J. V. *Acc. Chem. Res.* **1988**, *21*, 348.

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

12. Hajra, A. K.; Agranoff, B. W. *J. Biol. Chem.* **1968**, *243*, 1617.

13. Coulson, A. B. W.; Knowles, J. R. *J. Chem. Soc., Chem. Commun.* **1970**, 7.

14. Gefflaut, T.; Périé, J. *Synth. Commun.* **1993**, *24*, 29.

15. Gresham, T. L.; Jansen, J. E.; Shaver, F. W. *J. Am. Chem. Soc.* **1950**, *72*, 72.

16. Pfitzner, K. E.; Moffat, J. G. *J. Am. Chem. Soc.* **1965**, *87*, 5661.

17. Parikh, R. J.; Doering, W. E. *J. Am. Chem. Soc.* **1967**, *89*, 5505.

18. Segel, I. H. *Enzyme Kinetics: Behavior and Analysis of Steady-State and Rapid Equilibrium Enzyme Systems*; Wiley-Interscience: New York, 1975.

19. Santi, D. V.; Ouyang, T. M.; Tan, A. K.; Gregory, D. H.; Scanlan, T.; Carreras, C. W. *Biochemistry* **1993**, *32*, 11819.

20. Ginsburg, A.; Mehler, A. H. *Biochemistry* **1966**, *5*, 2623.

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

22. Fridovich, I. *J. Biol. Chem.* **1968**, *243*, 1043.

23. Tagaki, W.; Guthrie, J.; Weistheimer, F. *Biochemistry* **1968**, *7*, 905.

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

25. Wagner, J.; Lerner, R. A.; Barbas, III, C. F. *Science* **1995**, *270*, 1797.

26. Lai, C. Y.; Martinez de Dretz, G.; Bacila, M.; Marinello, E.; Horecker, B. L. *Biochem. Biophys. Res. Commun.* **1968**, *30*, 665.

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

28. Suh, B.; Barker, R. *J. Biol. Chem.* **1971**, *246*, 7041.

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

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

31. Kawahara, K.; Tanford, C. *Biochemistry* **1966**, *5*, 1578.

32. BIOSOFT, copyright 1991, 1992; PO Box 10938, Ferguson MO 63135, U.S.A.

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

(*Received in U.S.A.* 29 *April* 1996; *accepted* 23 *July* 1996)