32. Stereochemistry of the Inhibition of δ-Chymotrypsin with Optically Active Bicyclic Organophosphates: ^{31}P-NMR Studies

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The inhibition of δ-chymotrypsin with optically active, axially and equatorially substituted *trans*-3-(2,4-dinitrophenoxy)-2,4-dioxa-3λ^5-phosphabicyclo[4.4.0]decan-3-ones (= hexahydro-4*H*-1,3,2-benzodioxaphosphorin 3-oxides) was investigated. Their inhibitory power was determined by kinetic measurements, and the stereochemical course of the reaction of stoichiometric amounts of the enzyme and inhibitor was monitored with ^{31}P-NMR spectroscopy at pH 7.8. The irreversible inhibitors show significant enantioselectivity (the (S_P)-enantiomer reacting faster) and yield diastereoisomeric, covalently phosphorylated derivatives of δ-chymotrypsin.

^{31}P-NMR Spectroscopic studies of the inhibition by the axially substituted inhibitor revealed for the racemic (±)-**2a** first a resonance at −4.4 ppm and later, while inhibition proceeded, a second one at −4.5 ppm. The reaction with optically active (+)-**2a** showed only one signal at −4.4 ppm and its enantiomer (−)-**2a** only one signal at −4.5 ppm. Using the equatorially substituted racemic epimer (±)-**2b**, we observed the main resonance at −5.3 ppm and two minor ones at −4.4 and −4.5 ppm. The optically active compound (+)-**2b** showed two peaks at −4.5 and −5.3 ppm, whereas its antipode (−)-**2b** revealed two signals at −4.4 and −5.3 ppm.

Comparing the ^{31}P chemical shifts of the corresponding covalent phosphoserine derivatives **4a** (−5.7 ppm, axial) and **4b** (−4.5 ppm, equatorial) shows the inhibition with the axial compounds **2a** to proceed *via* neat inversion of the configuration at the P-atom, whereas the equatorial epimers **2b** with a higher conformational flexibility seem to follow a different stereochemical pathway which results in both inversion and retention.

Introduction. – The acute toxicity of organophosphorus compounds is due to their inhibitory action on acetylcholinesterase and related serine hydrolases such as chymotrypsin [1]. They react with the nucleophilic O–C(β) of the active-site serine residue in the catalytic triade (Asp^{102} ··· His^{57} ··· Ser^{195} in chymotrypsin [2]) in a displacement process at the P-atom. The covalently phosphorylated enzyme forms a stable, tetrahedral adduct which is considered to be a good analogue of the tetrahedral carbonyl addition intermediate or its transition state [3].

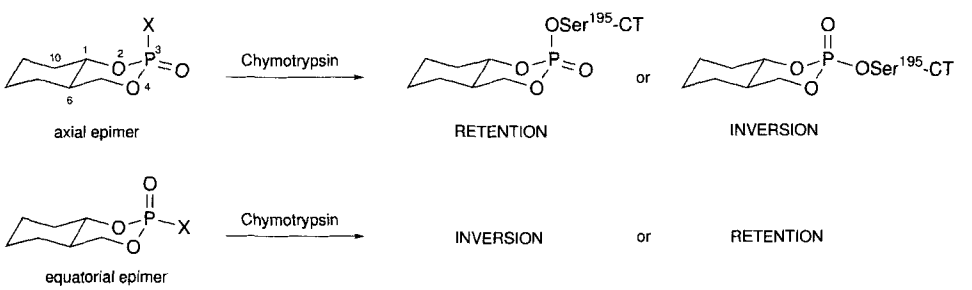
Therefore, organophosphates and -phosphonates represent unique active-site probes of serine hydrolases enabling ^{31}P-NMR spectroscopic studies of the irreversibly inhibited enzyme species [4]. As the ^{31}P chemical shift is very sensitive to the nature of the substituents and to configurational and conformational changes at the P-atom [5], both the regio- and the stereochemical course of the inhibition reaction can be investigated. Such experiments provide direct structural and mechanistic information on the active site of serine hydrolases. Especially the enzymatic stereochemistry of serine hydrolases is still of considerable interest, as not only the primary inhibitory reaction [6–11], but also postinhibitory phenomena [12–16] are currently studied. Moreover, as the enzymes are highly diastereo- and enantioselective, experiments with enantiomeric inhibitors [8–11] [15–17] give a deeper insight into more complex stereochemical implications. On the

other hand, the enzymes can be considered as 'stereochemical probes' in order to elucidate the course of nucleophilic displacement reactions at the P-atom.

No group of enzymes is as ubiquitous and as fundamental to a host of critical functions as the serine hydrolases and proteases [9] [18]. Since organophosphorus compounds are potent inhibitors of these enzymes, the exact knowledge of their mode of action is the prerequisite for the understanding of organophosphorus poisoning by insecticides or nerve agents and, as a consequence, its prophylaxis and therapy. These facts drew our attention to open questions concerning the regio- and stereochemistry of serine hydrolases.

In that respect, a pioneering paper of *Gorenstein* and coworkers [6] prompted us to prepare several novel 2,4-dioxa-3λ^5-phosphabicyclo[4.4.0]decan-3-ones (= hexahydro-4*H*-1,3,2-benzodioxaphosphorin 3-oxides) as inhibitors of serine hydrolases (chymotrypsin, acetylcholinesterase) and model compounds concerning their reaction mechanism [19]. Being configurationally and conformationally locked, these *trans*-decaline congeners are good probes for the investigation of stereochemical implications by ^{31}P-NMR spectroscopy. Several years ago, detailed conformational analyses of 2,4-dioxa-3λ^5-phosphabicyclo[4.4.0]decan-3-ones and related ring systems have been performed [20], and a set of spectroscopic arguments for the unequivocal assignment of their structures has been established [21][22][1]). Based on these results, the interpretation of the corresponding ^{31}P-NMR spectra would allow to determine the stereochemical course of the inhibition reaction as depicted in the general *Scheme 1*[2]).

Scheme 1



X = good leaving group, *e.g.* F, Cl, 4-nitrophenoxy, 2,4-dinitrophenoxy

Chymotrypsin (EC 3.4.21.1) is one of the best investigated enzymes [2] and is considered to be a good model for the cholinesterases. Moreover, it is readily available in high purity, and due to its molecular weight (25 kD) and solubility, it was expected to be

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[1]) The ^{31}P-NMR resonance of the axial epimer (X axial, see *Scheme 1*) is shifted upfield with respect to the equatorial one, and the splitting pattern (^3J(P, H)) in the ^{1}H-coupled ^{31}P-NMR is indicative of the conformation of the heterocyclic ring. Due to the anomeric effect, electronegative substituents X occupy the favoured axial position. As a consequence, axially substituted cyclic phosphates adopt the chair and its equatorial counterparts a twist-boat conformation. Moreover, the axial isomer mostly elutes faster in a chromatographic system.

[2]) *Gorenstein* and coworkers [6] demonstrated that the inhibition of α-chymotrypsin with a large excess of (±)-**2a** occurs with inversion of the configuration at the P-atom. The equatorial epimer (±)-**2b** was considered not to be a substrate for the enzyme.

suitable for direct ^{31}P-NMR measurements with stoichiometric amounts of the inhibitors. δ-Chymotrypsin was chosen to avoid the difficulties encountered with the dimerization of the α-species resulting in additional ^{31}P-NMR signals which impeded the interpretation [7] (see also [10])[3]). The 2,4-dinitrophenoxy-substituted inhibitors were selected because of their moderate inhibitory power (k_i *ca.* 10^3 $M^{-1}min^{-1}$) which allowed to monitor the course of the inhibition reaction on the NMR time scale as well as from the fact that both the axial and the equatorial epimers could be prepared[4]). In this paper, the optically active compounds are characterized for the first time.

Synthesis and Kinetic Characterization of the Inhibitors. – The optically active *trans*-3-(2,4-dinitrophenoxy)-2,4-dioxa-3λ^5-phosphabicyclo[4.4.0]decan-3-ones were prepared from the *trans*-configurated (+)-(1*S*,2*R*)- and (−)-(1*R*,2*S*)-2-(hydroxymethyl)cyclohexan-1-ols ((+)- and (−)-**1**, resp.) by reaction with 2,4-dinitrophenyl phosphorodichloridate and chromatographic separation of the resulting mixture (axial/equatorial *ca.* 1:1) into the pure axial epimers (+)-**2a** and (−)-**2a** and equatorial epimers (+)-**2b** and (−)-**2b** (*Scheme 2, Table*). Similarly, starting from (±)-**1**, the racemic substances [6] were obtained. Racemic (±)-**1** was prepared from 1,2-epoxycyclohexane according to [24], and the optically pure (+)- and (−)-*trans*-2-(hydroxymethyl)cyclohexan-1-ols ((+)-**1** and (−)-**1**, resp.) were obtained after resolution of the intermediate (±)-*trans*-2-hydroxycyclohexane-1-carboxylic acid with (−)-(*S*)- and (+)-(*R*)-(1-phenylethyl)amine, respectively (see *Exper. Part*).

The phosphoserine model compounds **4a** and **4b** were prepared from the phosphorochloridate (±)-**3** according to [6] (*Scheme 2*). The ^{31}P-NMR chemical shifts and the splitting pattern in the ^{1}H-coupled ^{31}P-NMR enabled the unambiguous assignment of all the epimers (see *Exper. Part*)[1]).

For the determination of the inhibitory power of the individual compounds, no direct kinetic method could be used. As the inhibition proceeds at a rather moderate rate, δ-chymotrypsin was incubated at pH 7.8 (25°) with an excess of inhibitor in the absence of substrate and the residual activity of the enzyme monitored by the BTEE assay[5]). Data interpretation was performed according to the procedure of [25] (see *Exper. Part*). Under the assumption that the inhibition is predominantly bimolecular and intermediate reactions insignificant, this method is applicable to slow, irreversible inhibitors. However, the calculated overall bimolecular reaction constants k_i are merely approximative; in particular, there is no information concerning the dissociation constant (K_D) and the rate of phosphorylation (k_p). The compounds are irreversible inhibitors, and the following k_i values ([$M^{-1}min^{-1}$]) were determined (*Table*): 1150 for (±)-**2a**, 1660 for (+)-**2a**, 220 for (−)-**2a**, 1020 for (±)-**2b**, 1350 for (+)-**2b**, 305 for (−)-**2b**. These results clearly demonstrate the significant enantioselectivity of the enzyme and its preference for the (S_p)-enantiomer[6]).

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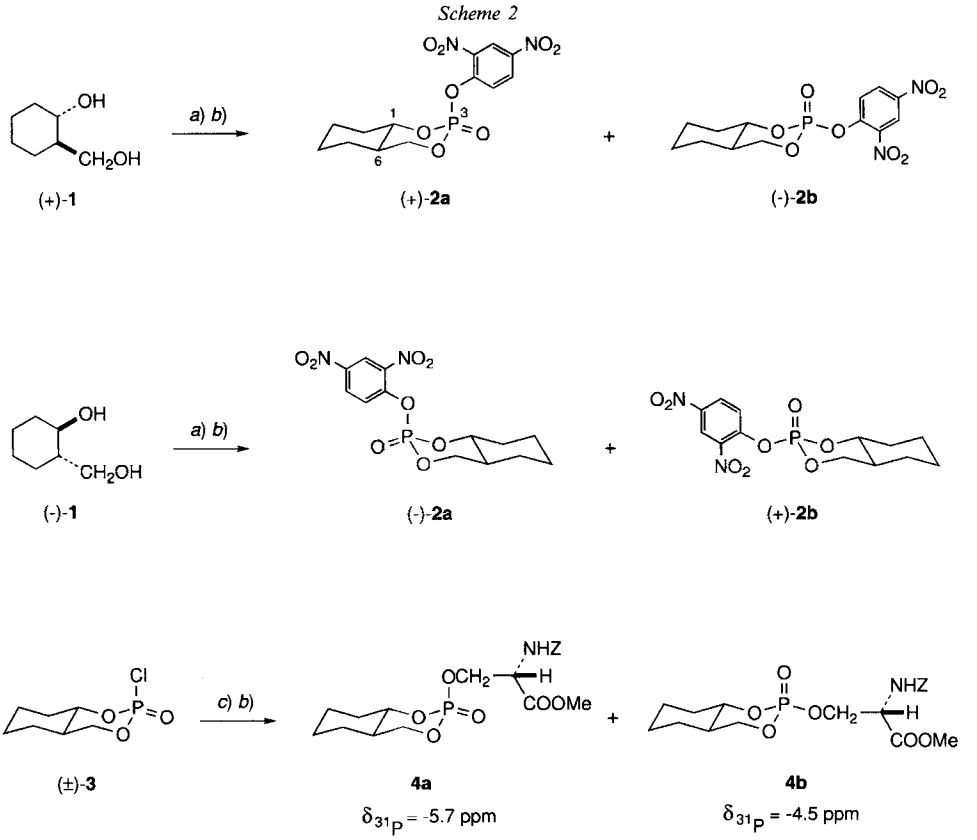
[3]) The most significant difference between α- and δ-chymotrypsin is that α-chymotrypsin dimerizes at pH < 7 by reaction of the free α-carboxylate of Tyr^{146} with His^{57} of a second molecule. δ-Chymotrypsin contains two additional amino acids (Thr^{147}-Asn^{148}) which render it incapable of dimerizing [23].

[4]) More electronegative substituents X (*e.g.* Cl) occupy exclusively the axial 3-position [21], less electronegative ones (*e.g.* 4-nitrophenoxy) proved to be too weak inhibitors [19].

[5]) BTEE = *N*-Benzoyl-L-tyrosine ethyl ester.

[6]) This result is consistent with the finding that the (S_p)-configuration seems to be preferred by serine hydrolases [17].

Scheme 2



a) 2,4-Dinitrophenylphosphorodichloridate, pyridine, $CHCl_3$, 0°, 6 h. *b*) Chromatography (SiO_2, hexane or $CHCl_3$/AcOEt). *c*) *N*-[(benzyloxy carbonyl])-L-serine methyl ester, pyridine, $CHCl_3$, 0°, 6 h.

Table. *Kinetic, Chiroptic, and* ^{31}*P-NMR Spectroscopic Data of the Inhibition of δ-Chymotrypsin with the* trans-*3-(2,4-Dinitrophenoxy)-2,4-dioxa-3λ*^{5}*-phosphabicyclo[4.4.0]decan-3-ones* **2a** *and* **2b**

Inhibitor	$[\alpha]_D^{20}$ [a])	Abs. configuration	k_i [$M^{-1}min^{-1}$]	δ [ppm] [b])	δ [ppm] [b]) after inhibition (assignment)			
(±)-**2a**			1150	−14.7	−2.4 (**5**)	−4.4 (**6**)	−4.5 (**6**)	
(+)-**2a**	+3.9	(1*S*,3S_P,6*R*)	1660	−14.7	−2.4 (**5**)	−4.4 (**6**)		
(−)-**2a**	−4.2	(1*R*,3R_P,6*S*)	220	−14.7	−2.4 (**5**)		−4.5 (**6′**)	
(±)-**2b**			1020	−14.1	−2.4 (**5**)	−4.4 (**6**)	−4.5 (**6′**)	−5.3 (**7**,**7′**)
(+)-**2b**	+47.0	(1*R*,3S_P,6*S*)	1350	−14.1	−2.4 (**5**)		−4.5 (**6′**)	−5.3 (**7**)
(−)-**2b**	−42.5	(1*S*,3R_P,6*R*)	350	−14.1	−2.4 (**5**)	−4.4 (**6**)		−5.3 (**7′**)

[a]) In $CHCl_3$, see comments in the *Exper. Part.*
[b]) In MeCN (11%)/D_2O (44%)/0.2M *Tris*, pH 7.8 (45%).

Results and Discusssion of the ^{31}P-NMR Investigations. – *General.* To monitor directly the progression of the reaction, the experiments were designed so that the enzyme and the inhibitors could react in stoichiometric amounts (*ca.* 2 μmol each in *Tris* buffer at pH 7.8 (25°))[7]). The inhibitor solutions gave clearly observable ^{31}P-NMR signals at −14.7 (**2a**) and −14.1 ppm (**2b**) already after a few scans. In all our experiments, after adding the enzyme solution, there was no signal detectable for a longer period (1–2 h)[8]), whereas later, a signal at −2.4 ppm developed rapidly. Scans accumulated in hourly intervals showed the progression of the slow inhibition reaction until a stationary state was reached after *ca.* 24 h. When eight-days-old samples of δ-chymotrypsin inhibited with (±)-**2a** and (±)-**2b** (left at room temperature) were examined by ^{31}P-NMR, they did not show any differences compared to the 24 h old samples. Hence, postinhibitory reactions [15] of the phosphoenzyme (*e.g.* aging [12–14]) were not observed under our experimental conditions.

Racemic and Optically Active 3-Axial Compounds **2a** *as Inhibitors.* When δ-chymotrypsin was inhibited with (±)-**2a**, the signal at −2.4 ppm was visible after *ca.* 1.5 h; after *ca.* 4 h, an additional signal at −4.4 ppm was observed and, later, while inhibition proceeded, a slowlier increasing second one at −4.5 ppm. After 24 h, both resonances had the same intensity (the most representative sections are shown in *Fig. 1*). The inhibition experiments with (+)-**2a** ((S_P)-configuration) revealed a single peak at −4.4 ppm (besides that at −2.4 ppm), whereas using (−)-**2a** ((R_P)-configuration) resulted in a single peak at −4.5 ppm (*Fig. 2*). The only difference that could be found was the significantly higher rate of peak formation with (+)-**2a** as inhibitor.

We assign the rapidly developing signal at −2.4 ppm to the hydrolysis product **5**. This peak could easily be removed by ultrafiltration of the sample (cutoff 10 kD) and the phosphoric acid which was prepared by controlled saponification of (±)-**2a** exhibited the same chemical shift. The formation of **5** is a consequence of the combined mechanism of action of the serine hydrolases which involves general acid-base and covalent catalysis [26][9]). Obviously, the two signals at −4.4 and −4.5 ppm (*Fig. 2*) represent two diastereoisomeric covalent phosphoenzymes, *i.e.*, **6** and **6′**. Moreover, comparison of the ^{31}P-NMR chemical shifts with the equatorially substituted model compound **4b** (−4.5 ppm) suggests that the Ser^{195} moiety of δ-chymotrypsin occupies the equatorial position at the P-atom (*Scheme 3*, *Table*). Hence, adduct formation proceeded with inversion of the configuration at the P-atom. This is explained in terms of an in-line S_N2(P) displacement process by apical attack (see **A**) of the activated O–C(β) of Ser^{195}

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[7]) In most of the inhibiton experiments described in the literature (see *e.g.* [6] [7] [13] [14]), the enzyme is treated with a large excess of the inhibitor followed by separation and isolation of the phosphoenzyme prior to spectroscopy. This approach restricts the investigation to the final state, and postinhibitory phenomena which might render the interpretation more difficult have also to be considered. There are only a few exceptions where stoichiometric amounts of inhibitor and enzyme have been applied and the ^{31}P-NMR spectra measured immediately [10] [12]. Most significantly, the generation of diastereoisomeric phosphonate ester adducts and different reaction rates for the enantiomers were evidenced for the first time [10]. However, an assignment of the respective absolute configurations was not possible.

[8]) We interpret this fact as a coalescence phenomenon which may represent the formation of the *Michaelis-Menten* complex and its dynamic behaviour.

[9]) In the absence of the enzyme, the *trans*-3-(2,4-dinitrophenoxy)-2,4-dioxa-3λ^5-phosphabicyclo[4.4.0]decan-3-ones are stable to hydrolysis under the reaction conditions.

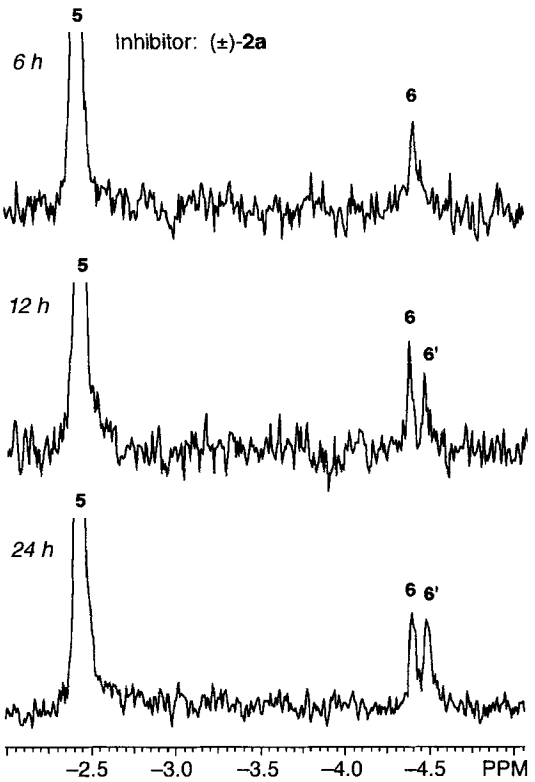


Fig. 1. *$^{31}P\{^1H\}$-NMR Spectra of representative periods of the inhibition of δ-chymotrypsin with racemic* (±)-**2a**. Signal at −2.4 ppm. hydrolysed inhibitor (**5**); at −4.4 ppm: diastereoisomeric phosphoenzyme **6** formed from the faster reacting (+)-**2a** ($k_i = 1660\text{M}^{-1}\text{min}^{-1}$); at −4.5 ppm: diastereoisomeric phosphoenzyme **6′** formed from the slower reacting (−)-**2a** ($k_i = 220\text{M}^{-1}\text{min}^{-1}$).

and apical departure [27] of the 2,4-dinitrophenoxy group from the pentacoordinated intermediate **B**, as depicted in *Scheme 4*.

The relative rate differences by which the adducts are formed are also reflected by the kinetic data. As (+)-**2a** is the faster-reacting inhibitor ($k_i = 1660\text{M}^{-1}\text{min}^{-1}$) the result that the signal at −4.4 ppm (**6**) increases faster than that at −4.5 ppm corresponding to **6′** formed from (−)-**2a** ($k_i = 220\text{M}^{-1}\text{min}^{-1}$) is consistent with the inhibition constants.

Racemic and Optically Active 3-Equatorial Compounds **2b** *as Inhibitors.* The analogous experiments with δ-chymotrypsin and the equatorial epimers **2b** were not as straightforward as those with the axial ones. In particular, when monitoring the time course of the inhibition, we observed transient broad peaks which could not be interpreted unambiguously. They are indicative of the dynamic behaviour of the system and are due to coalescence phenomena. Therefore, we have to restrict our discussion to the steady state which was observed after 24 h (*Fig. 3*). Using the racemic epimer (±)-**2b**, we found the main ^{31}P-NMR resonance at −5.3 ppm and, additionally, two minor ones at −4.4 and −4.5 ppm (very small[10])). The optically active compound (+)-**2b** gave rise to a

[10]) Only tentative assignment as the signal hardly exceeds the noise.

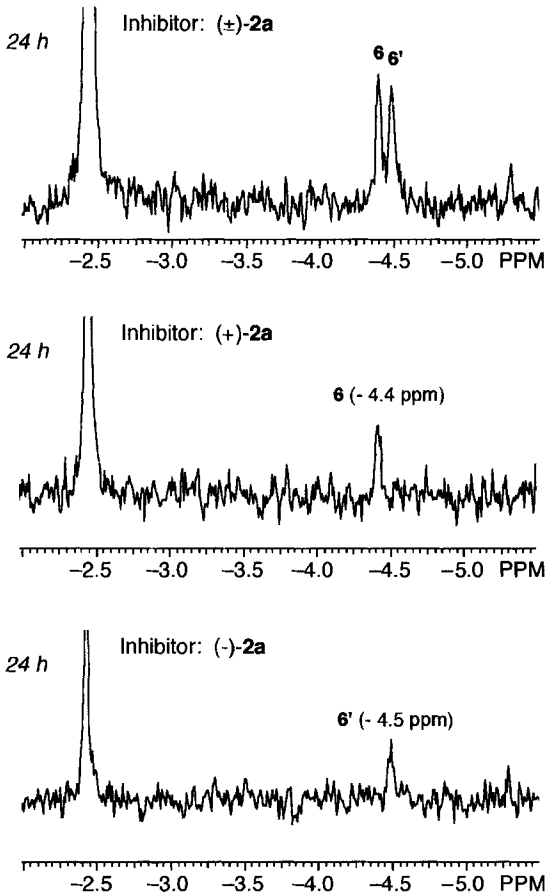
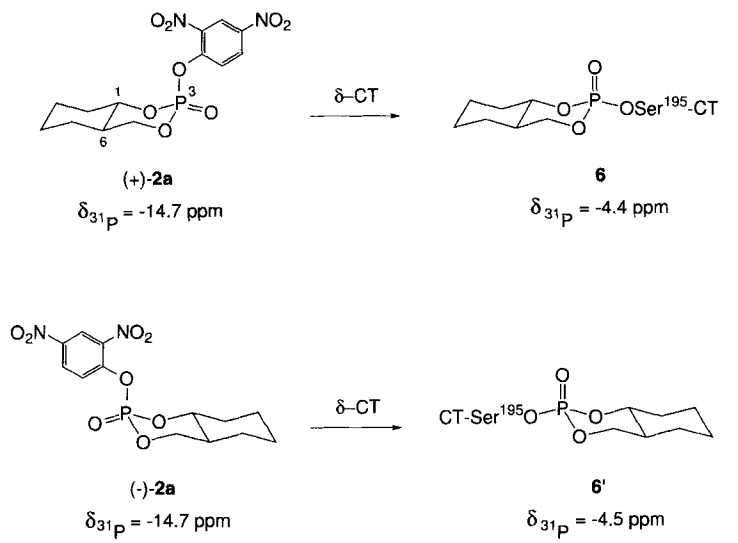


Fig. 2. *Comparison of the* $^{31}P\{^1H\}$*-NMR spectra after inhibition* (24 h) *of δ-chymotrypsin with the axial compounds* (±)-**2a** *and the optically active* (+)-**2a** *and* (−)-**2a**.

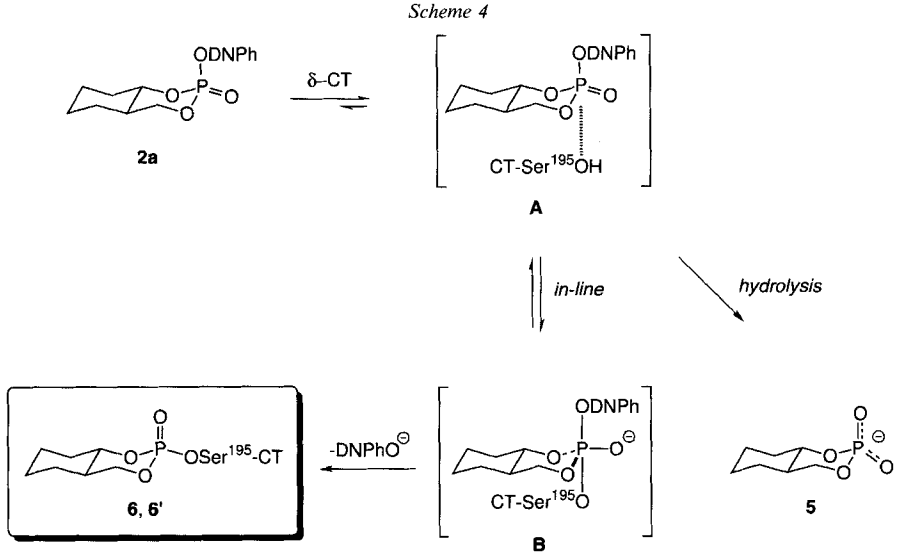
major peak at −5.3 and a minor signal at −4.5 ppm, whereas the antipode (−)-**2b** revealed two signals of almost equal intensity at −4.4 and −5.3 ppm. The hydrolysis product **5** (−2.4 ppm) was detected after *ca.* 2 h in all three experiments.

Comparison of the ^{31}P-NMR chemical shifts with the model compound **4a** (−5.7 ppm) suggests the main resonance (−5.3 ppm) to be representative of an axial Ser^{195} moiety in the phosphorylated δ-chymotrypsin. Hence, structures **7** and **7′** are assigned to the diastereoisomeric phosphoenzymes, and we conclude that the main compounds **7** and **7′** have been formed by inversion of the configuration at the P-atom (*Scheme 5*, *Table*). In contrast to their equatorial counterparts **6** and **6′**, the diastereoisomers **7** and **7′** have the same ^{31}P chemical shift and cannot be distinguished. However, the occurrence of the additional minor signals (−4.4 and/or −4.5 ppm) which are due to equatorial substitution patterns at the P-atom clearly demonstrate that also the phosphoenzymes **6** and **6′** have been formed. Obviously, the stereochemical course of this reaction is retention at the P-atom.

Scheme 3



Scheme 4



The experimental facts are rationalized in *Scheme 6*. For steric reasons, an in-line attack of the nucleophile opposite to the leaving group is unlikely in the chair conformer **2b**, merely an adjacent entry (see **C**) [27] [28] would be feasible. The favourable arrangement of the substituents in the intermediate **D** where the 2,4-dinitrophenoxy leaving group is already in the required apical position enables its facile departure without prior

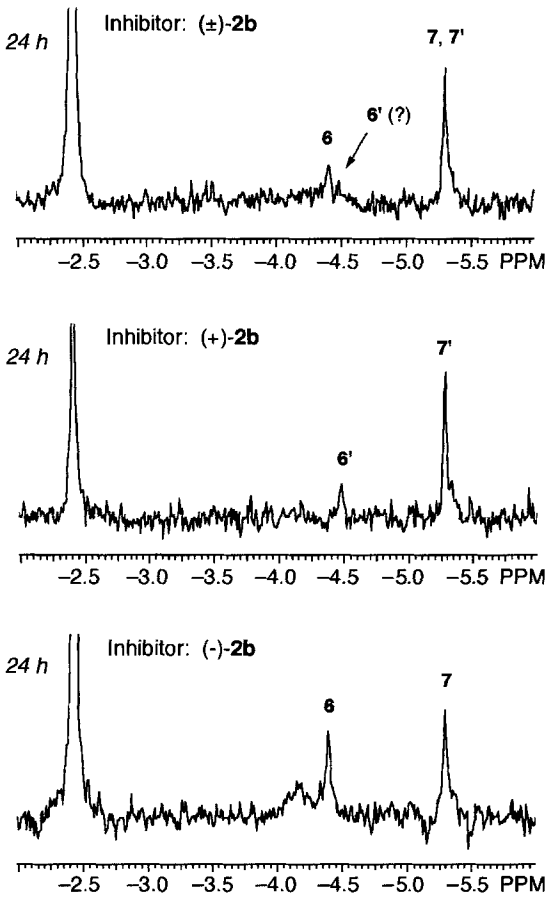
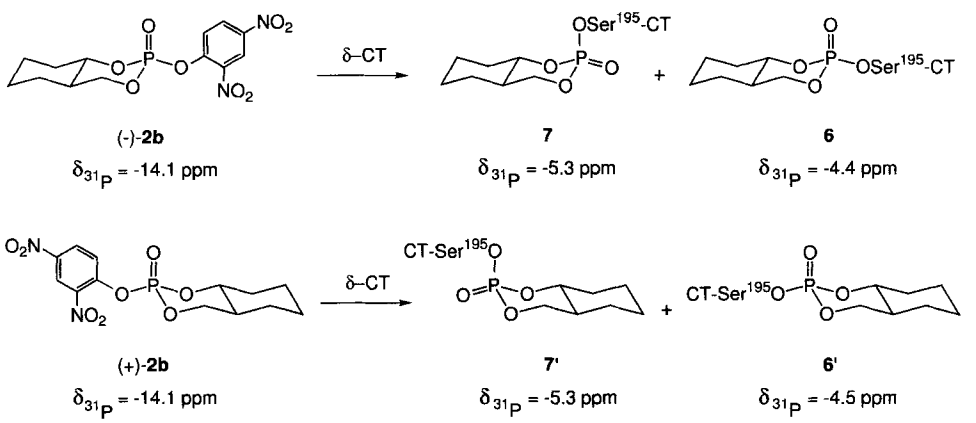


Fig. 3. *Comparison of the $^{31}P\{^1H\}$-NMR spectra after inhibition* (24 h) *of δ-chymotrypsin with the equatorial compounds (±)-***2b** *and the optically active (+)-***2b** *and (−)-***2b**

Scheme 5



(-)-**2b** $\delta_{^{31}P}$ = -14.1 ppm

7 $\delta_{^{31}P}$ = -5.3 ppm

6 $\delta_{^{31}P}$ = -4.4 ppm

(+)-**2b** $\delta_{^{31}P}$ = -14.1 ppm

7' $\delta_{^{31}P}$ = -5.3 ppm

6' $\delta_{^{31}P}$ = -4.5 ppm

pseudorotation[11]). The result of this process are the phosphoenzymes **6** and **6′** where the configuration at the P-atom is retained. According to stereoelectronic considerations which strongly favour the axial arrangement of an electronegative substituent, equatorially substituted cyclic phosphates [21] and phosphonates [22] preferentially adopt a twist-boat conformation[1])[12]). Therefore, the observed inversion can be explained by a direct *in-line displacement process* at **2b′** (*via* **E**) during which the twist-boat conformation in the trigonal bipyramid **F** may be maintained until the favourable chair is re-established from intermediate **G** yielding the axially substituted phosphoenzymes **7** and **7′**.

Remarks. – The mechanism of nucleophilic displacement reactions between phosphate esters and related compounds has been the subject of several significant investigations from which conflicting results have emerged[13]). In particular, exocyclic displacements occur with a bewildering variety of stereochemistries, dependent on the nature of the substrate, attacking nucleophile, leaving group, solvent, and added salts[14]). As a matter of fact, the stereochemistry of six-membered P-containing rings is still poorly understood [28].

Nonenzymic reactions of both five- and six-membered systems are comparable in wealth of mechanistic pathways available, including in-line and adjacent displacement mechanisms, pseudorotation of trigonal bipyramidal intermediates, and competing pathways. In contrast, it was stated by *Westheimer* (see in [28]) that all enzymic reactions at P proceed with inversion and, therefore, occur without pseudorotation. In fact, there is no unambiguous evidence that pseudorotation or adjacent attack at the P-atom is a process of significance in any biological system, and formal retention is rationalized by a multistep process with an even number of inversions [27] [28] [30][15]).

The results of our investigation with the equatorial substrates **2b** may be an example where an adjacent attack needs not be followed by pseudorotation, because the leaving group can move directly into the favourable apical position. However, these conclusions are to be treated as preliminary. Unless we are able to rationalize the dynamic behaviour

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[11]) In comparable, nonenzymic displacement reactions, retention at the P-atom is generally rationalized by an equatorial (adjacent) entry of the nucleophile followed by ligand reorganization (pseudorotation) according to their relative apicophilicities and apical departure of the leaving group [27] [28].

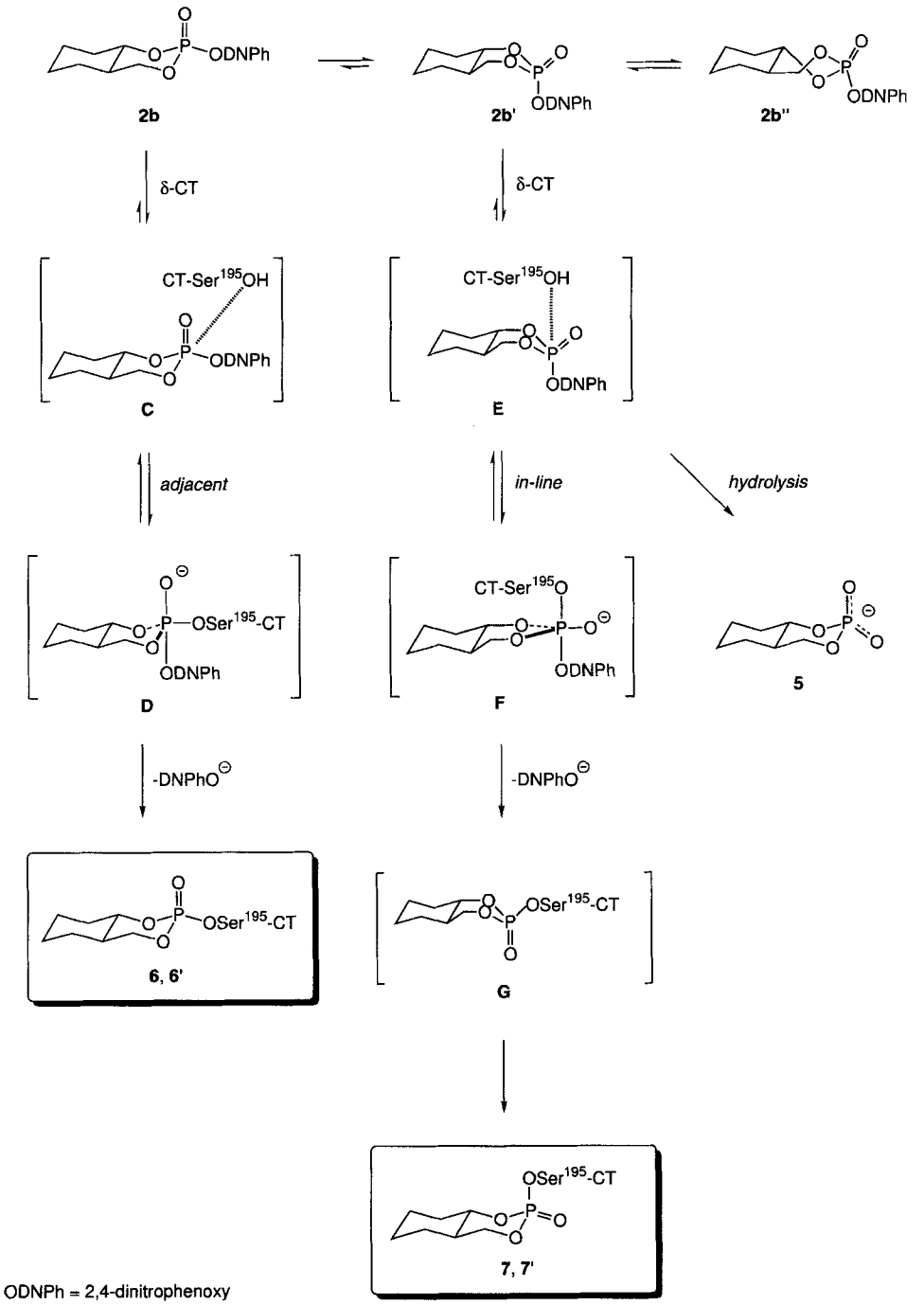
[12]) As can be concluded from the characteristic ^3J(P, H) splitting pattern in the ^{1}H-coupled ^{31}P-NMR, electronegatively substituted 3-equatorial 2,4-dioxa-3λ^5-phosphabicyclo[4.4.0]decan-3-ones exist in an equilibrium mixture of twist and boat conformers. Thus, by flipping from the heterocyclic chair **2b** to the twist-boat conformations **2b′** and **2b″** (*Scheme 6*), the chair equatorial ester bond in **2b** moves into a pseudoaxial position. These conformations represent a balance between the anomeric effect favouring the axial orientation in the twist-boat and the 1,3 steric and eclipsing interactions favouring the chair conformation, hence furnishing an explanation for the unusual stabilization of **2b′** and **2b″** [21].

[13]) For the most comprehensive, critical review covering the last 4 decades of research on that subject, see [28].

[14]) We recently demonstrated [29] that also the bulkiness of otherwise very similar auxiliary bases can be crucial in the stereochemical outcome of nucleophilic catalysed displacements at P.

[15]) Recently, it could be shown by X-ray analysis [9] that the phosphonylation of α-lytic protease by P-epimeric hexapeptide analogues yields the same covalent adduct. This result indicates inversion of configuration at P for one diastereoisomer and retention for the other one. Although the authors are well aware that displacement at P with retention through pseudorotation is unprecedented in enzymatic systems, they rather prefer it than a two-step mechanism [9].

Scheme 6



ODNPh = 2,4-dinitrophenoxy

of **2b** during inhibition, the pathway leading to retention at P cannot be fully understood [16]). Appropriate experiments to elucidate the coalescence phenomena are in course.

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Experimental Part

1. *General.* Abbrevations: BTEE = *N*-Benzoyl-L-tyrosine ethyl ester, *Tris* = tris(hydroxymethyl)aminomethane (= 2-amino-2-(hydroxymethyl)propane-1,3-diol). Reagents: $CHCl_3$ *puriss p.a.* (*Fluka AG*), filtered over *Alox 90* basic, act. I (*Merck*); δ-chymotrypsin (EC 3.4.21.1) *Sigma C-9381*, from bovine pancreas, essentially salt free, lot 82H8030, 40–80 U/mg solid [17]); AcOEt *purum*, distilled and then dried over molecular sieve 4 Å; hexane *purum*, distilled; H_2O, bidistilled; silica gel *60* 0.04–0.063 mm (*Merck*); pyridine *puriss p.a.* (*Fluka*); *Tris puriss. p.a.* (*Fluka*); $CDCl_3$ and D_2O, *Cambridge Isotope Laboratories*. Kinetic measurements: *Hewlett-Packard-8452A* diode array spectrophotometer using the *HP-89532A* kinetic software; the quartz cuvettes were thermostatted (25°) in a *Julabo U3* water bath. M.p.: *Mettler FP5/FP52*; uncorrected. $[\alpha]_D^{20}$: *Perkin-Elmer-241-MC* polarimeter with thermostat *B. Braun Thermomix 1441*, 10-cm cell. ^{1}H-NMR: *Bruker AC-300* (300 MHz), chemical shifts δ in ppm rel. to the assigned solvent (Me_4Si = 0 ppm), coupling constants *J* in Hz, $w_{1/2}$ = line width at half-height. ^{13}C-NMR: *Bruker ARX-300* (75.4 MHz). All assignments are based on ^{1}H,^{1}H- and ^{1}H,^{13}C-COSY and DEPT 135 spectra. ^{31}P-NMR: *Bruker AC-300* (121.4 MHz) or *Bruker AM-400-WB* (161.9 MHz), 85% H_3PO_4 as external standard. Electro-spray ionization (ESI) MS: *Finningan TSQ 700*.

2. *(±)-trans-3-(2,4-Dinitrophenoxy)-2,4-dioxa-3λ⁵-phosphabicyclo[4.4.0]decan-3-ones* ((±)-**2a** and (±)-**2b**). To a soln. of (±)-**1** (187 mg, 1.4 mmol), pyridine (116 µl, 1.4 mmol), and $CHCl_3$ (3 ml) prepared at 0° in a glove-box under N_2, a soln. containing 2,4-dinitrophenyl phosphorodichloridate [18]) (432 mg, 1.4 mmol) in $CHCl_3$ (2 ml) was added dropwise. The mixture was stirred for 6 h at 0° and then evaporated. The yellow precipitate was dissolved in AcOEt, and the epimers were separated by column chromatography (silica gel *60*, hexane/AcOEt 3:2). From the faster eluting fraction, we isolated (±)-**2a** (92 mg, 18%) [19]) as colourless crystals and from the slower eluting one (±)-**2b** (55 mg, 11%) as slightly yellow crystals.

(±)-**2a**: M.p. 124–127°. R_f (hexane/AcOEt 3:2) 0.46. ^{1}H-NMR (300 MHz, $CDCl_3$): 1.07 (*dq*, $^2J \approx {}^3J$ (7ax,6) $\approx {}^3J$(7ax,8ax) $\approx$ 12, ^3J(7ax,8eq) = 3.5, H_{ax}–C(7)); 1.35 (br. *m*, *quint.*-like, H_{ax}–C(8), H_{ax}–C(9)); 1.60 (*d'q'*, $^2J = {}^3J$(10ax,1) = 10.5, ^3J(10ax,9ax) = 11, ^3J(10ax,9eq) = 3.7, H_{ax}–C(10)); 1.77 (br. *m*, *tt*-like, H_{eq}–C(7), H_{eq}–C(8)); 1.91 (*m*, *d*-like, H_{eq}–C(9)); 2.09 (br. *m*, *dt*-like, H–C(6)(*X* of *ABX-P*), H_{eq}–C(10)); 4.29 (*B* of *ABX-P*, ^2J = 10.5, ^3J(5ax,6) = 11, ^3J(5ax,P) ≈ 0, H_{ax}–C(5)); 4.38 (*dt*, ^3J(1,10ax) = ^3J(1,6) = 10.5, ^3J(1,10eq) = 4.8, H–C(1)); 4.33 (*A* of *ABX-P*, ^2J = 10.5, ^3J(5eq,6) = 4.6, ^3J(5eq,P) = 25, H_{eq}–C(5)); 8.12 (*dd*, ^3J(6',5') = 9.2, ^5J(6',3') = 1, H–C(6')); 8.46 (*dd*, ^3J(5',6') = 9.2, ^4J(5',3') = 2.7, H–C(5')); 8.83 (*dd*, ^4J(3',5') = 2.7, ^5J(3',6') = 1, H–C(3')). ^{13}C-NMR (75.4 MHz, $CDCl_3$): 23.8 (*d*, ^4J(9,P) = 2.5, C(9)); 24.1 (C(8)); 25.1 (C(7)); 32.3 (*d*, ^3J(10,P) = 8.7, C(10)); 40.8 (*d*, ^3J(6,P) = 6.2, C(6)); 74.0 (*d*, ^2J(5,P) = 7.7, C(5)); 84.8 (*d*, ^2J(1,P) = 7.5, C(1)); 121.6 (C(3')); 122.9 (C(6')); 129.1 (C(5')); 140.0 (C(2')); 143.3 (C(4')); 148.0 (*d*, ^2J = 4.5, C(1')). ^{31}P-NMR (121.4 MHz, ^{1}H-coupled, $CDCl_3$): −14.5 (*d*, ^3J(P,H_{eq}–C(5)) = 25). ^{31}P{^{1}H}-NMR (161.9 MHz, MeCN (11%)/D_2O (44%)/0.2M *Tris*, pH 7.8 (45%)): −14.7. ESI-MS: 381 ([M^+Na]$^+$).

(±)-**2b**: M.p. 109–112°. R_f (hexane/AcOEt 3:2) 0.24. ^{1}H-NMR (300 MHz, $CDCl_3$): 1.04 (*dq*, $^2J \approx {}^3J$ (7ax,6) $\approx {}^3J$(7ax,8ax) $\approx$ 12, ^3J(7ax,8eq) = 3.5, H_{ax}–C(7)); 1.35 (br. *m*, *quint.*-like, H_{ax}–C(8), H_{ax}–C(9)); 1.56

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[16]) During storage of the compounds, very slow epimerizations (−)-**2b** → (+)-**2a** and (+)-**2b** → (−)-**2a** are observed. Therefore, an alternative explanation could be an enzyme-catalysed epimerization to the thermodynamically preferred axial inhibitors (**2a**) prior to covalent bond formation. The different intensities of the ^{31}P-NMR signals of the adducts **6** and **6'** (*Fig. 3*) might also be indicative of the relative inhibition rates where the corresponding k_i values (*Table*) would be consistent. For a mechanistic explanation, see [31].

[17]) After several months of storage at −20°, the preparation showed an activity of 78 U/mg solid, according to the *Sigma* procedure. After one day at room temperature, the enzyme solution still showed an activity of 76 U/mg solid.

[18]) The crystalline product prepared according to [32] consists of $Cl_2P(O)OC_6H_3(NO_2)_2$ (85%; ^{31}P-NMR: 5.0 ppm) and $ClP(O)[OC_6H_3(NO_2)_2]_2$ (15%; ^{31}P-NMR: −6.5 ppm).

[19]) The low yields are due to decomposition of the labile compounds on silica gel during column chromatography.

(*d'q'*, $^2J = {}^3J(10\,ax,1) = 10.6$, $^3J(10\,ax,9\,ax) \approx 12$, $^3J(10\,ax,9\,eq) = 3.7$, H_{ax}–C(10)); 1.79 (br. *m*, H_{eq}–C(7), H_{eq}–C(8)); 1.91 (*m*, *d*-like, H_{eq}–C(9)); 2.20 (*m*, *d*-like, H_{eq}–C(10)); 2.34 (*X* of *ABX-P*, H–C(6)); 4.21 (*B* of *ABX-P*, $^2J = 10.5$, $^3J(5\,ax,6) = 10.5$, $^3J(5\,ax,P) = 11$, H_{ax}–C(5)); 4.37 (*ddt*, $^3J(1,10\,ax) = {}^3J(1,6) = 10.6$, $^3J(1,10\,eq) = 4.4$, $^3J(1,P) = 2.8$, H–C(1)); 4.46 (*A* of *ABX-P*, $^2J = 10.5$, $^3J(5\,eq,6) = 5.2$, $^3J(5\,eq,P) = 11.5$, H_{eq}–C(5)); 7.97 (*dd*, $^3J(6',5') = 9.2$, $^5J(6',3') = 1$, H–C(6')); 8.44 (*dd*, $^3J(5',6') = 9.2$, $^4J(5',3') = 2.7$, H–C(5')); 8.79 (*dd*, $^4J(3',5') = 2.7$, $^5J(3',6') = 1$, H–C(3')). ^{13}C-NMR (75.4 MHz, $CDCl_3$): 23.8 (C(9)); 24.0 (C(8)); 26.2 (C(7)); 32.6 (*d*, $^3J(10,P) = 6.3$, C(10)); 39.2 (*d*, $^3J(6,P) = 12.5$, C(6)); 74.3 (*d*, $^2J(5,P) = 7.5$, C(5)); 84.9 (*d*, $^2J(1,P) = 6.8$, C(1)); 121.4 (C(3')); 123.8 (C(6')); 128.8 (C(5')); 140.7 (C(2')); 143.5 (C(4')); 148.2 (*d*, $^2J(1',P) = 4.5$, C(1')). ^{31}P-NMR (121.4 MHz, ^{1}H-coupled, $CDCl_3$): –13.8 (*t*, $^3J(P, H_{ax}–C(5)) = 11$, $^3J(P, H_{eq}–C(5)) = 11.5$). $^{31}P\{^1H\}$-NMR (161.9 MHz, CH_3CN (11%)/D_2O (44%)/0.2M *Tris*, pH 7.8 (45%)): –14.1. ESI-MS: 381 ($[M^+Na]^+$).

3. *(+)-(1S,2R)- and (–)-(1R,2S)-2-(Hydroxymethyl)cyclohexan-1-ols* ((+) and (–)-**1**, resp.). Resolution of (±)-*trans*-2-hydroxycyclohexane-1-carboxylic acid (prepared according to [24] from 1,2-epoxycyclohexane *via trans*-2-cyanocyclohexan-1-ol) with (–)-(*S*)-(1-phenylethyl)amine yielded (+)-(1*S*,2*S*)-2-hydroxycyclohexane-1-carboxylic acid ($[\alpha]_D^{20} = +54.1$ (*c* = 0.46, $CHCl_3$)) and with (+)-(*R*)-(1-phenylethyl)amine (–)-(1*R*,2*R*)-2-hydroxycyclohexane-1-carboxylic acid ($[\alpha]_D^{20} = -51.0$ (*c* = 1.02, $CHCl_3$))[20]) was obtained. Reduction of the (+)- and (–)-enantiomer with $LiAlH_4$ in Et_2O gave (+)-**1** ($[\alpha]_D^{20} = +35.0$ (*c* = 1.19, EtOH)) and (–)-**1** ($[\alpha]_D^{20} = -35.1$ (*c* = 0.31, EtOH)), resp.[21]).

4. *(+)-(1S,3S$_P$,6R)- and (–)-(1S,3R$_P$,6R)-3-(2,4-Dinitrophenoxy)-2,4-dioxa-3λ^5-phosphabicyclo[4.4.0]decan-3-ones* ((+)-**2a** and (–)-**2b**). In analogy to the procedure for the synthesis of (±)-**2a,b**, starting from (+)-**1** (197 mg, 1.5 mmol), we obtained (+)-**2a** (70 mg, 13%) and (–)-**2b** (43 mg, 8%) both as colourless crystals. (+)-**2a**: m.p. 110–111°. $[\alpha]_D^{20} = +3.9$ (*c* = 1.00, $CHCl_3$). (–)-**2b**: m.p. 108–110°. $[\alpha]_D^{20} = -42.5$ (*c* = 1.00, $CHCl_3$). ^{1}H, ^{13}C, and ^{31}P-NMR and EI-MS: identical to those of (±)-**2a** and (±)-**2b**, resp.

5. *(–)-(1R,3R$_P$,6S)- and (+)-(1R,3S$_P$,6S)-3-(2,4-Dinitrophenoxy)-2,4-dioxa-3λ^5-phosphabicyclo[4.4.0]decan-3-ones* ((–)-**2a** and (+)-**2b**). Starting from (–)-**1** (170 mg, 1,3 mmol), we obtained (–)-**2a** (67 mg, 14%) as colourless crystals and (+)-**2b** (85 mg, 18%) as slightly yellow crystals. (–)-**2a**: m.p. 110–112°. [α] = –4.2 (*c* = 1.00, $CHCl_3$). (+)-**2b**: m.p. 108–109°. $[\alpha]_D^{20} = +47.0$ (*c* = 1.00, $CHCl_3$). ^{1}H, ^{13}C, and ^{31}P-NMR and EI-MS: identical to (±)-**2a** and (±)-**2b**, resp.

6. *(±)-N-[(Benzyloxy)carbonyl]-*O*-(*trans*-3-oxo-2,4-dioxa-3λ^5-phosphabicyclo[4.4.0]dec-3-yl)-*L*-serine Methyl Ester* (**4a** and **4b**). According to [6], reaction of (±)-*trans*-3-chloro-2,4-dioxa-3λ^5-phosphabicyclo[4.4.0]decan-3-one ((±)-**3**; 5.40 g, 25 mmol, prepared from (±)-**1** with $POCl_3$) and *N*-[(benzyloxy)carbonyl]-L-serine methyl ester (6.55 g, 26 mmol) yielded, after chromatography (silica gel, $CHCl_3$/AcOEt 1:1), the faster eluting 3-axial epimer **4a** (4.57 g, 43%) as colourless crystals followed by the 3-equatorial epimer **4b** (0.53 g, 5%) as a colourless viscous oil.

4a: M.p. 123–125.5°. ^{1}H-NMR (300 MHz, $CDCl_3$): 0.88 (br. *m*, *q*-like, H_{ax}–C(7')); 1.26 (br. *m*, *t*-like, H_{ax}–C(8'), H_{ax}–C(9')); 1.50 (*d'q'*, $^2J \approx {}^3J(10'\,ax,1') \approx {}^3J(10'\,ax,9'\,ax) \approx 11$, $^3J(10'\,ax,9'\,eq) \approx 4$, H_{ax}–C(10')); 1.6–2.1 (br. *m*, H–C(6') (*X* of *ABX-P*), H_{eq}–C(7'), H_{eq}–C(8'), H_{eq}–C(9'), H_{eq}–C(10')); 3.80 (*s*, MeO); 3.87 (*B* of *ABX-P*, $^2J \approx {}^3J(5'\,ax,6') \approx 11$, $^3J(5'\,ax,P) \approx 0$, H_{ax}–C(5')); 3.94 (*dt*, $^3J(1',10'\,ax) \approx {}^3J(1',6') \approx 11$, $^3J(1',10'\,eq) = 4.5$, H–C(1')); 4.12 (*A* of *ABX-P*, $^2J = 11$, $^3J(5'\,eq,6') = 4.2$, $^3J(5'\,eq,P) = 23$, H_{eq}–C(5')); 4.37–4.50 (*AB* of *ABX-P*, not resolved, CH_2(3)); 4.62 (*X* of *ABX-P*, not resolved, *d*-like, H–C(2)); 5.14 (*s*, PhCH_2); 5.83 (br. *d*, $^3J(NH, 2) = 7.5$, NH–C(2)); 7.31–7.37 (*m*, 5 arom. H). ^{13}C-NMR (75.4 MHz, $CDCl_3$): 23.8 (C(9')); 24.2 (C(8')); 25.1 (C(7')); 32.3 (*d*, $^3J(10',P) = 9.0$, C(10')); 40.7 (*d*, $^3J(6',P) = 5.9$, C(6')); 52.8 (MeO); 54.5 (*d*, $^3J(2',P) = 6.7$, C(2)); 66.5, 66.6 (*d*, $^2J(3,P) = 5.1, 4.9$, C(3)[22]); 67.1 (PhCH_2); 72.8 (*d*, $^3J(5',P) = 6.0$, C(5')); 83.1, 83.2 (*d*, $^2J(1',P) = 6.6$, C(1'))[22]); 128.0, 128.1, 128.4 (arom. CH); 136.0 (arom. C); 155.7 (OCON); 169.5 (C(1)). ^{31}P-NMR (121.4 MHz, ^{1}H-coupled, $CDCl_3$): –7.0 (*dt*, $^3J(P, H_{eq}–C(5')) = 23$, $^3J(P, CH_2(3)) = 6$). $^{31}P\{^1H\}$-NMR (161.9 MHz, MeCN (11%)/D_2O (44%)/0.2M *Tris*, pH 7.8 (45%)): –5.7.

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[20]) As the resolution according to literature procedures [33] gave unsatisfactory results, we modified it by using (+)-(*R*)- and (–)-(*S*)-(1-phenylethyl)amine as chiral selectors.

[21]) Due to solvation effects and the conformational mobility of the hydroxymethyl group, the $[\alpha]_D$ values of **1** are strongly dependent on the solvent and the temperature [34]: $[\alpha]_D^{25} = +72$ (*c* = 0.4, H_2O) and $[\alpha]_D^{25} = +13$ (*c* = 0.4, CH_2ClCH_2Cl) for (+)-**1** and $[\alpha]_D^{25} = -55$ (*c* = 0.34, H_2O) for (–)-**1** (!). A full account on the determination of the optical purities (ee) and the absolute configurations of 3-substituted 2,4-dioxa-3λ^5-phosphabicyclo[4.4.0]decan-3-ones and their precursors as well as the difficulties connected therewith will be published elsewhere [35].

[22]) Double signals due to diastereoisomerism.

4b: ^{1}H-NMR (300 MHz, $CDCl_3$): 0.94 (br. *m*, *q*-like, H_{ax}–C(7')); 1.27 (br. *m*, *t*-like, H_{ax}–C(8'), H_{ax}–C(9')); 1.45 (br. *m*, *q*-like, H_{ax}–C(10')); 1.6–2.0 (br. *m*, 5H, H_{eq}–C(7'), H_{eq}–C(8'), H_{eq}–C(9'), H_{eq}–C(10')); 2.12 (*m*, $w_{1/2} \approx 20$, H–C(6') (*X* of *ABX-P*)); 3.79 (*s*, MeO); 4.07–4.28 (*AB* of *ABX-P*, not resolved, and *m*, H_{ax}–C(5'), H_{eq}–C(5'), H–C(1')); 4.33–4.62 (*ABC* of *ABC-P*, not resolved, H–C(2), CH_2(3)); 5.13 (*s*, PhCH_2); 5.73 (br. *s*, NH–C(2)); 7.31–7.37 (*m*, 5 arom. H). ^{13}C-NMR (75.4 MHz, $CDCl_3$): 23.8 (*d*, ^4J(9',P) = 2.1, C(9')); 24.2 (C(8')); 25.6 (C(7')); 32.6 (*d*, ^3J(10',P) = 8.2, C(10')); 40.76, 40.81 (*d*, ^3J(6',P) = 6.3, 6.7, C(6'))[22]; 52.8 (MeO); 54.3 (*d*, ^3J(2,P) = 6.7, C(2); 67.1 (PhCH_2); 67.87, 67.92 (2*d*, ^2J(3,P) = 5.5, 6.0, C(3))[22]; 72.2 (*d*, ^3J(5',P) = 5.6, C(5')); 82.1 (*d*, ^2J(1,P) = 5.5, C(1')); 128.0, 128.1, 128.4 (arom. CH); 136.0 (arom. C); 155.7 (OCON); 169.1 (C(1)). ^{31}P-NMR (121.4 MHz, ^{1}H-coupled, $CDCl_3$): −4.3 (*m*, $w_{1/2} \approx 36$). ^{31}P{^{1}H}-NMR (161.9 MHz, MeCN (11%)/D_2O (44%)/0.2M *Tris*, pH 7.8 (45%)): −4.5.

7. *Kinetic Experiments. Solutions.* Buffer: 0.1M *Tris* · HCl, pH 7.8/0.1M $CaCl_2$; substrate: BTEE (1 mM in H_2O/MeOH 95:5); enzyme stock soln.: δ-chymotrypsin ($4 \cdot 10^{-5}$ M in 0.001N HCl); inhibitor: $4 \cdot 10^{-3}$ M in dry MeCN.

Incubation (stock solution). Buffer (15 ml), δ-chymotrypsin stock soln. (300 µl), and MeCN (550 µl) were thoroughly mixed in a 50-ml polyethylene tube and partitioned into 3 portions (5 ml each); the residual 850 µl were used for the determination of the enzyme activity without inhibitor (v_0). To 5 ml of this stock mixture, inhibitor soln. (25 µl) was added and incubated at 25°. At discrete intervals, 100-µl aliquots were taken and the residual activity of the enzyme monitored by BTEE assay (v_t).

BTEE Assay. BTEE (1.5 ml) and buffer soln. (1.4 ml) were thermostatted at 25° for 5 min, then the incubated enzyme/inhibitor soln. (100 µl) was added and the turnover of the substrate monitored at λ 260 nm in a thermostatted quartz cell (l = 1 cm) at 25° for 3 min (the final concentrations of the solns. in the cuvette were: 0.5 mM BTEE, 2.5 vol.-% MeOH, 0.05M *Tris* · HCl (pH 7.8), 0.05M $CaCl_2$, $6 \cdot 10^{-7}$ M inhibitor).

Calculation. For the determination of k_i, at least 3 assays each furnishing 4–5 data points were performed and the values averaged. Linear regression of the plot of the slopes of the tangents drawn to the individual progress curves (v_0, v_t) against time at constant [I] yields the graph $\ln(v_0/v_t) = k_i[I] \cdot t$ from which the bimolecular reaction constant k_i is calculated (k_i = slope/[I]) [25].

8. *^{31}P-NMR Experiments. Sample Preparation.* Soln. *A*: the inhibitor (0.8 mg, 2.2 µmol) was dissolved in abs. MeCN (60 µl). Soln. *B*: δ-chymotrypsin (48 mg, 1.9 µmol) was dissolved in D_2O (240 µl) and *Tris* buffer (250 µl, pH 7.8, 0.2M). Soln. *A* was quickly added by use of a micropipette to soln. *B* and then transferred to a 5-mm NMR tube. The ^{31}P-NMR experiments were run immediately after mixing.

^{31}P-NMR Parameters. Bruker AM-400-WB, 161.9 MHz, triple resonance inverse probe head, temperature (300 ± 1 K); ^{31}P chemical shifts in ppm rel. to 85% H_3PO_4 as external reference; pulse width 8 µs (45°); relaxation delay 1 s.; acquisition time 0.72 s, spectral width 11000 Hz; line-broadening factor 2.0 Hz.

Time Program. At the beginning of inhibition two spectra of 2000 transients were recorded (*ca.* 1 h each), then spectra with 4000 transients were recorded (*ca.* 2 h each). For better signal to noise ratio, several FIDs (*f*ree *i*nduction *d*ecays) consecutively measured in the time period of interest were added and the desired spectra obtained by *Fourier* transformation.

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