Stereochemistry of the Inhibition of δ-Chymotrypsin with Optically Active *cis*-Decaline-Type Organophosphates: ^{31}P-NMR Studies

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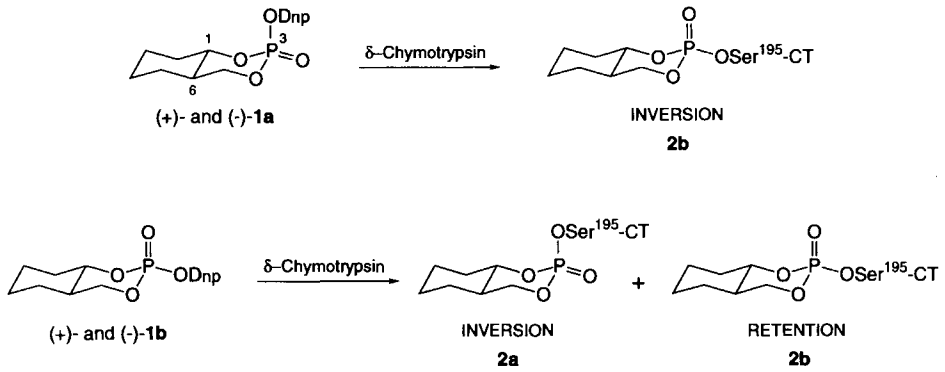
Investigation of the inhibition of δ-chymotrypsin with the four novel, optically active, axially and equatorially substituted *cis*-3-(2,4-dinitrophenoxy)-2,4-dioxa-3λ^5-phosphabicyclo[4.4.0]decan-3-ones (= 3-(2,4-dinitrophenoxy)hexahydro-4*H*-1,3,2-benzodioxaphosphorin 2-oxides) showed only the equatorially substituted enantiomer (–)-**4b** to be an irreversible inhibitor of the enzyme. ^{31}P-NMR Spectroscopic monitoring of the inhibition of stoichiometric amounts of the enzyme and (–)-**4b** at pH 7.8 revealed a quickly rising resonance at –2.49 ppm assigned to the hydrolysis product **8** and later, while inhibition proceeded, a second one at –4.08 ppm, attributed to the δ-chymotrypsin adduct **7** (*Scheme 3*). Comparison of the latter signal with the ^{31}P-NMR chemical shifts of the covalent phosphoserine model compounds (–)-**6a** (–5.67 ppm, axial substitution) and (+)-**6b** (–4.02 ppm, equatorial substitution) suggests that the inhibition proceeded *via* neat retention of the configuration at the P-atom of (–)-**4b** yielding the equatorially substituted covalent Ser^{195} adduct **7**.

Introduction. – In the course of our current program concerning the synthesis of conformationally restricted organophosphates as inhibitors of serine hydrolases and the investigation of the regio- and stereochemistry of the inhibition reaction, we have recently reported on the enantiomeric *trans*-3-(2,4-dinitrophenoxy)-2,4-dioxa-3λ^5-phosphabicyclo[4.4.0]decan-3-ones (= 3-(2,4-dinitrophenoxy)hexahydro-4*H*-1,3,2-benzodioxaphosporin 2-oxides) **1a** and **1b** [1][1]). It could be demonstrated that these configurationally and conformationally locked *trans*-decaline congeners follow different stereochemical pathways in the stoichiometric inhibition of δ-chymotrypsin (CT): The axially substituted epimers **1a** react *via* neat inversion, whereas their equatorially substituted counterparts **1b** show both inversion and retention of the configuration at the P-atom, yielding the covalent Ser^{195} adducts **2a** and **2b**, respectively (*Scheme 1*). Moreover, the reaction is significantly enantioselective, the (S_P)-enantiomers reacting faster. In continuation of our efforts on that topic, we present the results of the corresponding novel *cis*-decaline-type isomers (+)- and (–)-**4a** and (+)-and (–)-**4b**, respectively.

Synthesis and Characterization of the Inhibitors. – The optically active *cis*-3-(2,4-dinitrophenoxy)-2,4-dioxa-3λ^5-phosphabicyclo[4.4.0]decan-3-ones were obtained from the *cis*-configurated (+)-(1*S*,2*S*)- and (–)-(1*R*,2*R*)-2-(hydroxymethyl)cyclohexan-1-ols ((+)- and (–)-**3**, resp.) by reaction with 2,4-dinitrophenyl phosphorodichloridate and chromatographic separation of the resulting mixture (axial/equatorial *ca.* 1:1) into the pure axially substituted epimers (+)-**4a** and (–)-**4a** and equatorially substituted epimers (+)-**4b** and (–)-**4b** (*Scheme 2*). The optically pure starting alcohol (+)-**3** was prepared

[1]) For a full account on the background of the project, its context, and pertinent references, see [1].

Scheme 1



ODnp = 2,4-dinitrophenoxy

by reduction of ethyl 2-oxocyclohexanecarboxylate with bakers' yeast [2], saponification of the resulting (+)-ethyl (1*R*,2*S*)-2-hydroxycyclohexanecarboxylate and reduction with $LiAlH_4$. Resolution of (±)-*cis*-2-hydroxycyclohexane-1-carboxylic acid (prepared by controlled reduction of ethyl 2-oxocyclohexanecarboxylate ($NaBH_4$) and saponification of the resulting (±)-ethyl 2-hydroxycyclohexanecarboxylate) with brucine [3] and reduction of the (−)-(1*S*,2*R*)-2-hydroxycyclohexane-1-carboxylic acid ($LiAlH_4$) gave the starting alcohol (−)-**3** (see *Exper. Part*).

The epimeric phosphoserine model compounds (−)-**6a** and (+)-**6b** were prepared from (−)-**3** and *in situ* generated *N*-[(benzyloxy)carbonyl]-*O*-(dichlorophosphinyl)-L-serine methyl ester (**5**) and obtained pure after chromatographic separation (*Scheme 2*)[2]).

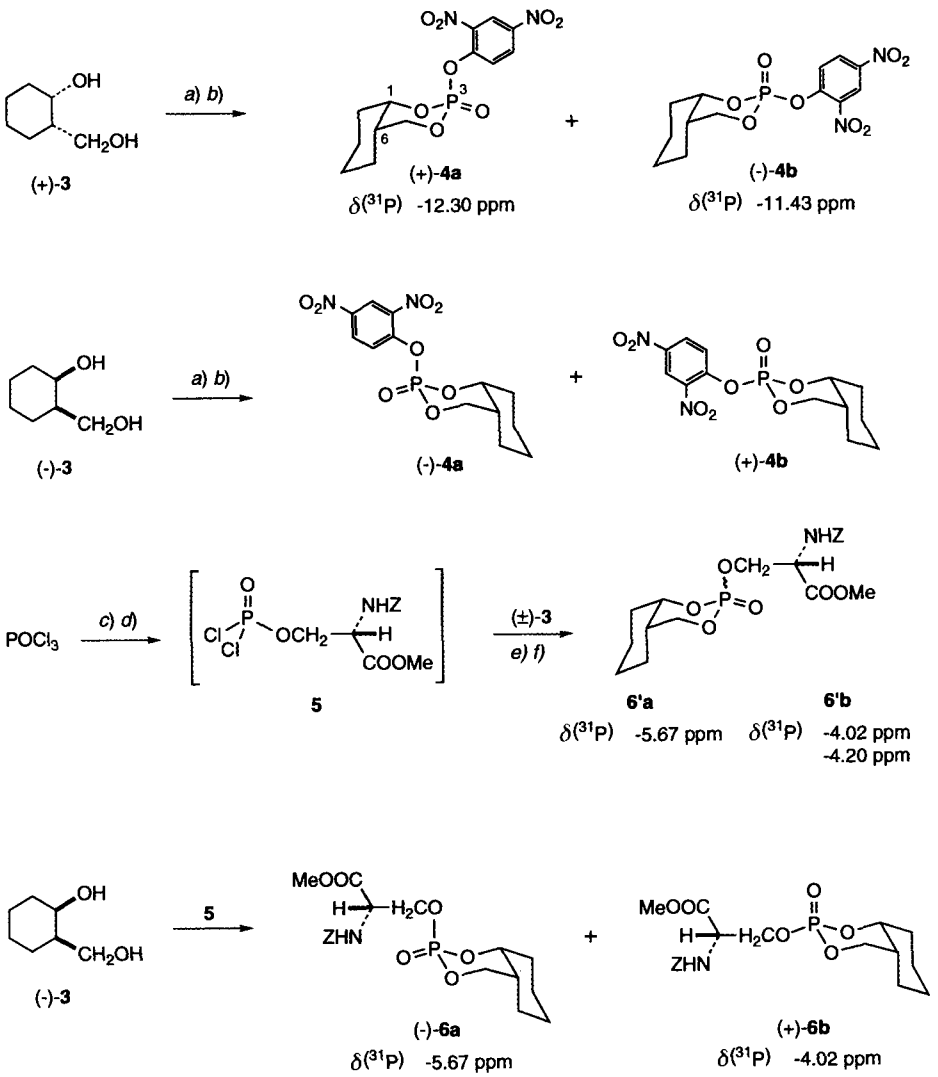
As the P(3)-substituted *cis*-2,4-dioxa-3λ^5-phosphabicyclo[4.4.0]decan-3-ones **4a**, **4b**, **6a**, and **6b** were prepared for the first time, a thorough spectroscopic characterization was indispensable[3]). Although the conformation of the *cis*-decalines is more flexible than that of the *trans*-isomers[4]), it could be shown that the same spectroscopic arguments

[2]) The reaction of (±)-**3** with **5** yielded the epimers **6'a** and **6'b** both as a mixture (*ca.* 1:1) of diastereoisomers. For an unambiguous assignment of the ^{31}P-NMR resonances, the enantiomerically pure diastereoisomers (−)-**6a** and (+)-**6b** were prepared.

[3]) Until now, the only related *cis*-decaline which has been characterized is (±)-6-methyl-3-(4-nitrophenoxy)-2,4-dioxa-3λ^5-phosphabicyclo[4.4.0]decan-3-one (axially substituted epimer, $\delta(^{31}P)$ −12.9 ($CDCl_3$)) [4]. The equatorially substituted epimer was not separated from the corresponding *trans*-isomer, and there are no spectral data available.

[4]) Only the equatorially substituted epimers **4b** exhibited coalescence phenomena in the ^{1}H-NMR spectra (see *Exper. Part*). Due to the conformational flexibility, a facile epimerization (*e.g.*, (−)-**4b** ⇌ (+)-**4a**) which could elapse in the NMR time scale is *a priori* to be expected. However, until now, we have no evidence for such a process to take place under the NMR conditions [7], see also the *Figure* (left). However, the equatorially substituted compounds epimerize when not worked up carefully and during storage (see also *Exper. Part*).

Scheme 2



Z = *N*-[(benzyloxy)carbonyl]

a) 2,4-Dinitrophenyl phosphorodichloridate, pyridine, $CHCl_3$, 0° → 20°, 12 h. *b*) Chromatography (SiO_2, hexane/Et_2O 2:3 *RP-188*, CH_2Cl_2). *c*) Pyridine, Et_2O, 0°. *d*) *N*-[(Benzyloxy)carbonyl]-L-serine methyl ester, Et_2O, 0°, 2 h. *e*) Pyridine, Et_2O, 0° → 20°, 12 h. *f*) Chromatography (SiO_2, Et_2O/MeOH 50:1).

concerning the configuration at the P-atom and the conformation in the heterocyclic ring are valid as those established in the *trans*-series [1][4–6]: Due to the dependence of the ^{31}P-NMR chemical shift on P–O torsional angles, axially substituted esters are generally expected to resonate upfield with respect to the equatorially substituted

epimers[5]), and the splitting pattern (^3J(P,H)) in the ^{1}H-coupled ^{31}P-NMR is indicative of the conformation of the heterocyclic ring[6]). Moreover, the anomeric preference which drives electronegative substituents into the stereoelectronically favoured axial position is so strong that equatorially substituted esters adopt a twist-boat conformation[7]). On behalf of these arguments, the spectral data (see *Exper. Part*) led to the unambiguous assignment of the relative configurations of the novel substances. Similarly to the observations in the *trans*-decaline series, all the *cis*-compounds which had an upfield ^{31}P-NMR shift also displayed a large ^3J(P,H) (*ca.* 25 Hz; *d*); therefore, the axial configuration was attributed to them. In contrast, the respective downfield-shifted resonances of their counterparts were accompanied by complex spin systems in the ^{1}H-coupled ^{31}P-NMR spectra, and they were assigned as the equatorially substituted epimers. Like in the *trans*-series [1], also the axially substituted *cis*-epimers eluted faster in the chromatographic systems.

The kinetic measurements which were performed in analogy to the procedure in [1] showed that (±)-**4a** did not inhibit δ-chymotrypsin (see below, *Scheme 3*), whereas the equatorially substituted isomer (±)-**4b** was shown to be a slow irreversible inhibitor ($k_i < 100\text{M}^{-1}\,\text{min}^{-1}$)[8]). Hence, only the enantiomers (+)- and (−)-**4b** were investigated further by ^{31}P-NMR spectroscopy.

Results and Discussion of the ^{31}P-NMR Investigations. – Similarly to the preceding studies [1], the experiments were designed such that the enzyme δ-chymotrypsin and the equatorially substituted potential inhibitors (+)- and (−)-**4b** could react in stoichiometric amounts (*ca.* 2 μmol each in *Tris* buffer at pH 7.8 (25°)). This procedure allows the

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[5]) According to stereoelectronic considerations, a phosphate diester in a *gauche,gauche* conformation would have a ^{31}P-NMR chemical shift substantially upfield from a phosphate ester in more extended conformations such as *gauche,trans* or *trans,trans* (*trans* = *anti*-periplanar) [6]. In the equatorially substituted epimers **4b** and **6b**, the ester group is locked into a *trans* conformation (C–O–P–OR dihedral angle (Φ) = 180°) relative to the endocyclic P–O ester bond, resulting in a downfield shift compared to the axially substituted *gauche* esters **4a** and **6a** (Φ = 60°).

[6]) The magnitude of the vicinal ^3J(P,H) is dependent on the P–O–C–H dihedral angle (Φ) showing a maximum value at 180° and a broad minimum at *ca.* 90° (modified *Karplus* equation); therefore, the ^{1}H-coupled ^{31}P-NMR spectra are a valuable probe for the conformational analysis of cyclic phosphates [6]: In the chair conformation of the decaline-type compounds, only H_{eq}–C(5) has Φ = 180° resulting in a large coupling ($^3J \approx 25$ Hz), whereas H–C(1) and H_{ax}–C(5) have Φ = 60° showing only very small couplings ($^3J \approx 0$–1 Hz). As a consequence, a pseudo-*d* is observed in the ^{31}P-NMR. In other conformations, as Φ(P,H_{eq}–C(5)) decreases and Φ(P,H_{ax}–C(5)) and Φ(P,H–C(1)) increase, the ^{31}P-NMR spectra exhibit complex multiplicities reflecting twist-boat conformations (in a pure boat, Φ(P,H–C(1)), Φ(P,H_{ax}–C(5)), and Φ(P,H_{eq}–C(5)) ≈ 120° with similar $^3J \approx 10$–15 Hz and a pseudo-*q* would be observed). Moreover, in contrast to the *trans*-decalines, a ^4J(P,H_{eq}–C(10)) = 4.5 Hz was detected by thorough homo- and hetero-correlated 2D ^{1}H-, ^{13}C-, and ^{31}P-NMR experiments.

[7]) In such systems, the resulting minimum-energy 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. This fact explains the unusual stabilization of the twist-boat conformations.

[8]) The enzyme was incubated at pH 7.8 (25°) with an excess of potential inhibitor in the absence of substrate and the residual activity of the enzyme monitored by the BTEE assay (*N*-benzoyl-L-tyrosine ethyl ester). For data interpretation *etc.*, see [1]. As the numeric results with the racemic mixture (±)-**4b** were not satisfactorially reproducible, the individual k_i values of (+)-**4b** and (−)-**4b** have not been determined.

monitoring of the progression of the inhibition reaction directly by ^{31}P-NMR spectroscopy[9]). Selected $^{31}P\{^{1}H\}$-NMR spectra of the experiments with (+)- and (−)-**4b** are depicted in the *Figure*.

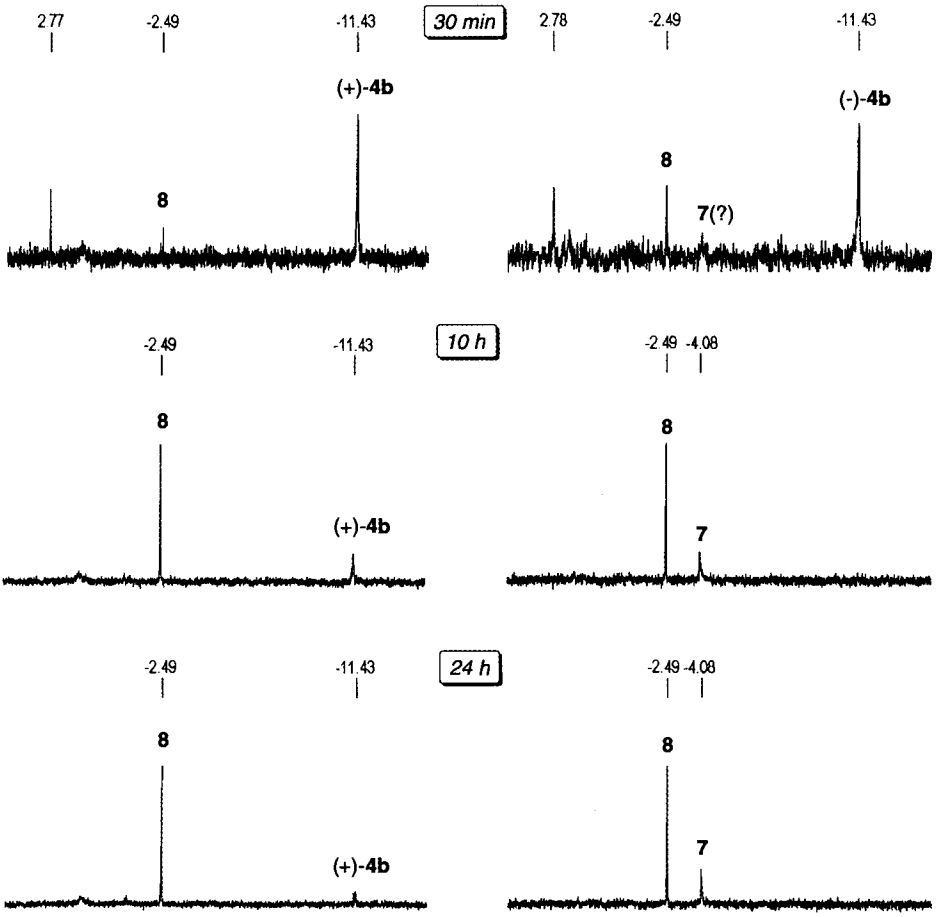


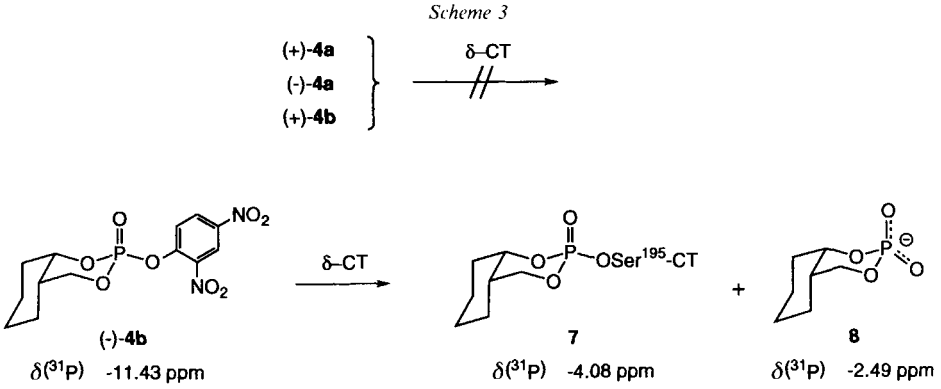
Figure. *$^{31}P\{^{1}H\}$-NMR Spectra* (242.9 MHz, 27°) *of the mixture obtained after representative periods of exposure of δ-chymotrypsin to the enantiomeric* cis*-decaline-type phosphates* (+)-**4b** (left) *and* (−)-**4b** (right). Signals at −2.49 ppm: hydrolysis product **8** (its enantiomer, resp.); at −4.08 ppm: equatorially substituted phosphoenzyme **7**; at −11.43 ppm: unreacted phosphates (+)-**4b** or (−)-**4b**, resp. The transient signal at 2.77 and 2.78 ppm could not be interpreted. Solvent: 0.2M *Tris* buffer (pH 7.8)/D_2O/MeCN 55:44:11.

[9]) In most of the comparable experiments described in the literature, the enzyme is treated with a large excess of the inhibitor followed by separation and isolation of the phosphoenzyme prior to ^{31}P-NMR spectroscopy [8][9]. This approach restricts the investigation to the final state, and postinhibitory phenomena which could render the interpretation more difficult must also be considered. There are only a few exceptions where stoichiometric amounts of inhibitor and enzyme have been applied and the spectra measured immediately [10].

Thus, when δ-chymotrypsin was added to a solution of (+)-**4b**, the signal of (+)-**4b** was immediately detected at −11.43 ppm[10]); after 30 min, additional resonances appeared at 2.77 and −2.49 ppm (see *Fig.*, top left); as the reaction proceeded, the peak at 2.77 ppm quickly disappeared (*ca.* 1 h) and that of (+)-**4b** constantly decreased until it was just visible after 24 h. When the analogous experiment was performed with δ-chymotrypsin and (−)-**4b**, the spectra recorded after 30 min (see *Fig.*, top right) resembled much those obtained in the experiment with (+)-**4a**, the only differences being a more intense signal at −2.49 ppm and a probably emerging resonance at *ca.* −4 ppm which hardly exceeded the noise. Scans accumulated in hourly intervals confirmed the proceeding of an inhibition reaction: after *ca.* 10 h, the peak of (−)-**4b** had disappeared, and a resonance at −4.08 ppm constantly increased until a stationary state was reached after *ca.* 24 h.

We assign the rapidly developing signal at −2.49 ppm in the mixtures obtained from both (+)- and (−)-**4b** to the hydrolysis product **8** (*Scheme 3*), since the phosphoric-acid derivative which was prepared by controlled saponification of (±)-**4b** exhibited almost the same chemical shift (−2.54 ppm). The formation of **8** is a consequence of the combined mechanism of action of the serine hydrolases which involves general acid-base and covalent catalysis [11]. The transient peak at 2.78 ppm could not be interpreted.

Scheme 3



Comparison of the $\delta(^{31}P)$ of the mixture obtained from (−)-**4b** with those of the model compounds (−)-**6a** (−5.67 ppm) and (+)-**6b** (−4.20 ppm) suggested that the resonance at −4.08 ppm arose from a covalent phosphoenzyme where the Ser^{195} moiety of δ-chymotrypsin occupies the equatorial position at the P-atom. Hence, the inhibition reaction proceeded with retention of the configuration at the P-atom, and the equatorially substituted covalent Ser^{195} adduct **7** was formed.

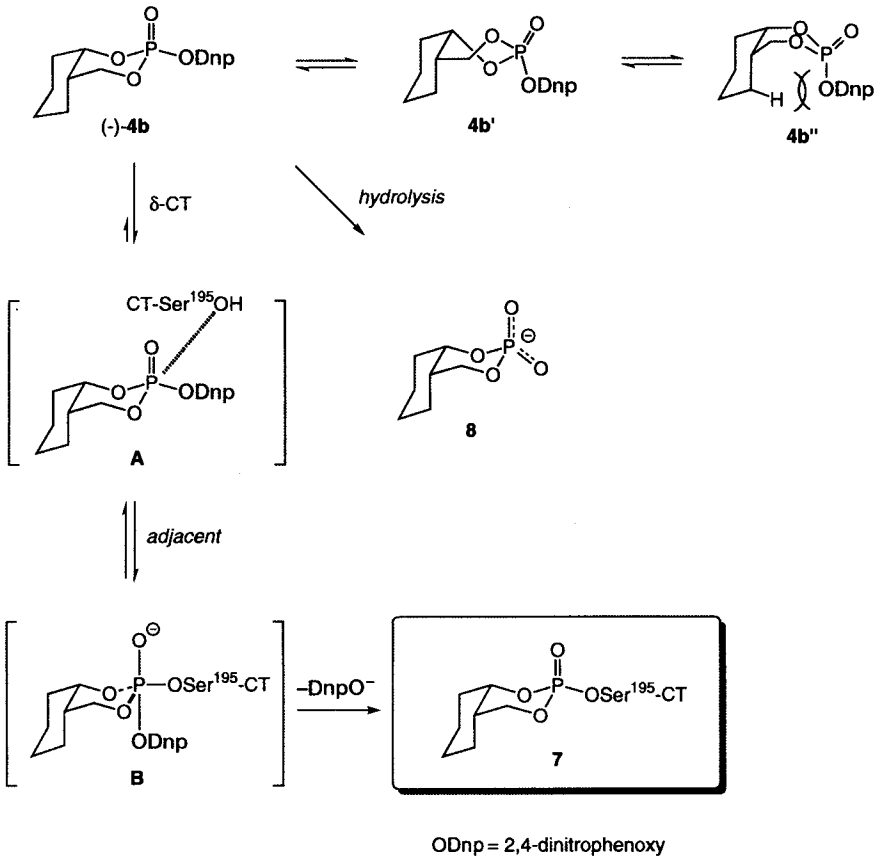
Obviously, the enantiomer (+)-**4b** is not an irreversible inhibitor of δ-chymotrypsin, but the inhibition studies (*Fig.*, left) established its relative stability towards hydrolysis under the experimental conditions. Moreover, there was no evidence of an enzyme-

[10]) This finding is different from those in similar experiments with the corresponding *trans*-decaline-type phosphates **1a** and **1b**, where the inhibitors never exhibited signals, and significant resonances of reaction products could be detected only after *ca.* 1–2 h [1].

catalysed epimerization to the axially substituted epimer (–)-**4a** which would exhibit a high-field $\delta(^{31}P)$ (12.30 ppm)[4]).

The experimental results (see *Scheme 3*) are rationalized in *Scheme 4*: For steric reasons, an $S_N2(P)$ displacement process by in-line attack of the activated O-atom at C(3) of Ser^{195} opposite to the leaving group is unlikely in the chair conformer (–)-**4b**, only an adjacent entry (see **A**) [12][13] would be feasible. The favourable arrangement of the substituents in the intermediate trigonal bipyramid **B**, where the 2,4-dinitrophenoxy leaving group is located in the required apical position, enables its facile departure without prior pseudorotation. The result of this process is the phosphoenzyme **7** where the configuration at the P-atom is retained[11]). The retention product **7** is also expected if a plausible adjacent attack of the nucleophile at the conformers **4b′** and **4b″** occurs.

Scheme 4



[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 [12][13].

Because of the anomeric preference which favours the axial arrangement of the electronegative dinitrophenoxy substituent [4–6], the twist-boat conformers **4b′** and **4b″** (*Scheme 4*) are expected to be present in the equilibrium, and an in-line attack of Ser^{195} at **4b″** and – eventually – at **4b′** could *a priori* be conceivable, too. Such an $S_N2(P)$ displacement process would proceed *via* inversion at the P-atom resulting in an axially substituted covalent Ser^{195} adduct, as it has been found with the *trans*-3-(2,4-dinitrophenoxy)-2,4-dioxa-3λ^5-phosphabicyclo[4.4.0]decan-3-ones (**1a** and **1b**) and explicitely explained in [1]. However, as no trace of a corresponding high-field ^{31}P-NMR signal could be detected (see *Fig.*), the experimental results show that the in-line pathway should be ruled out[12]).

Remarks. – Even though the mechanism of nucleophilic reactions of phosphate esters and related compounds has been the subject of many significant investigations in the past, the stereochemical course of exocyclic displacements at six-membered P-containing rings remains poorly understood [13][13]). Nonenzymic reactions of both five- and six-membered systems are found to occur by various mechanistic courses, including in-line and adjacent displacement mechanisms, pseudorotation of trigonal bipyramidal intermediates, and competing pathways. As a consequence, many results are equivocal, and the resulting stereochemistry is dependent on the nature of the substrate, attacking nucleophile, leaving group, solvent, and extrinsic factors[14]). In contrast, it was stated by *Westheimer* (see [13]) that all enzymic reactions at the P-atom proceed with inversion and, therefore, occur without pseudorotation. Indeed, there seems to be no unambiguous evidence that pseudorotation or adjacent attack at the P-atom is a significant process in biological systems until now, and formal retention is rationalized by a multistep process with an even number of inversions [12][13][15]).

The results of our investigations demonstrate that similar inhibitors under exactly the same reaction conditions show different stereochemical outcomes ranging from neat inversion (axially substituted *trans*-compounds (±)-, (+)-, and (−)-**1a** [1]) over mixed pathways (equatorially substituted *trans*-compounds (±)-, (+)-, and (−)-**1b**, [1]) to neat retention (*cis*-compound (−)-**4b**, this work) as an overall process. With regard to the statement of *Westheimer* concerning retention in enzymic reactions, the latter result is another example where the adjacent attack is not necessarily followed by pseudorotation as the leaving group directly moves into the favourable apical position. But we

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[12]) The ^{1}H-coupled ^{31}P-NMR spectrum (*q* at −14.2 ppm, with $^3J(P,H-C(1)) \approx {}^3J(P,H_{ax}-C(5)) \approx {}^3J(P,H_{eq}-C(5)) \approx 11.5$; in $CDCl_3$) suggests that (−)-**4b** adopts predominantly the boat conformation **4b″**. It can be assumed that the situation is probably different in the enzymic reaction where the eclipsing effects of the P(3)-substituted *cis*-2,4-dioxa-3λ^5-phosphabicyclo[4.4.0]decan-3-ones might become more significant than the stereoelectronic ones so that not all conformers may fit into the active site.

[13]) The review [13] constitutes the most comprehensive, critical paper covering the last 4 decades of research on that subject.

[14]) We recently have found that also the bulkiness of very similar auxiliary bases determines the stereochemical course of nucleophilic displacements at six-membered phosphate esters [14].

[15]) It has been demonstrated that the phosphonylation of α-lytic protease by P-epimeric hexapeptide analogues yields the same covalent adduct [15]. This fact is explained by inversion of the configuration at the P-atom for one diastereoisomer and retention for the other one. The authors are aware that displacement processes with pscudorotation at the P-atom are unprecedented in enzymic systems, nevertheless, they consider it to be more plausible than a two-step mechanism.

consider these conclusions still to be tentative as the real reaction pathways may be more complex than the experimental results make apparent. In this respect, the following observation seems to be significant: When an NMR sample of δ-chymotrypsin inhibited with (−)-**4b** was reinvestigated after 15 and 44 days, its ^{31}P-NMR showed a significant decrease of the peak at −4.08 ppm (**7**) and additional peaks were detected at −0.80 and −5.33 ppm. Hence, contrary to the inhibition results obtained with the *trans*-3-(2,4-dinitrophenoxy)-2,4-dioxa-3λ^5-phosphabicyclo[4.4.0]-decan-3-ones (**1a** and **1b**) [1], postinhibitory reactions [16] are probable in the case of (−)-**4b**. In a tentative interpretation, the low-field signal at −0.80 ppm may be attributed to an aged (dealkylated) phosphoenzyme [9], whereas the high-field resonance at −5.33 ppm could be assigned to an axially configurated phosphoenzyme (δ − 5.67 for the model compound (−)-**6a**). The latter might be due to a postinhibitory epimerization of the phosphoenzyme **7**. Appropriate experiments to elucidate these phenomena are in course.

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Experimental Part

1. *General.* See [1].

2. *(+)-(1S,2S)- and (−)-(1R,2R)-2-(Hydroxymethyl)cyclohexan-1-ol* ((+)- and (−)-**3**, resp.). Reduction of ethyl 2-oxocyclohexanecarboxylate with bakers' yeast according to [2], saponification of the resulting (+)-ethyl (1*R*,2*S*)-2-hydroxycyclohexanecarboxylate ($[\alpha]_D^{20}$ = + 26.1 (*c* = 0.75, $CHCl_3$)) and reduction with $LiAlH_4$ in Et_2O gave (+)-**3** ($[\alpha]_D^{20}$ = + 32.2 (*c* = 0.9, EtOH)).

Resolution of (±)-*cis*-2-hydroxycyclohexane-1-carboxylic acid (prepared by reduction of ethyl 2-oxocyclohexanecarboxylate with $NaBH_4$ in EtOH at 0°[16]) and saponification of the resulting (±-ethyl 2-hydroxycyclohexanecarboxylate) with brucine according to [3] and reduction of the (−)-(1*S*,2*R*)-2-hydroxycyclohexane-1-carboxylic acid ($[\alpha]_D^{20}$ = − 29.7 (*c* = 1.08, $CHCl_3$)) with $LiAlH_4$ in Et_2O gave (−)-**3** ($[\alpha]_D^{20}$ = − 31.8 (*c* = 0.93, EtOH))[17][18]).

3. *(±)-cis-3-(2,4-Dinitrophenoxy)-2,4-dioxa-3λ^5-phosphabicyclo[4.4.0]decan-3-ones* ((±)-**4a** and ±-**4b**). To a soln. of (±)-**3** (107 mg, 0.82 mmol) and pyridine (133 µl, 1.64 mmol) in $CHCl_3$ (1.5 ml), prepared at 0° in a glove-box under N_2, a soln. containing 2,4-dinitrophenyl phosphorodichloridate[19]) (297 mg, 0.99 mmol) in $CHCl_3$ (1.5 ml) was added dropwise. The mixture was stirred for 12 h at 20° and then evaporated. Fast CC of the residue (silica gel, activated overnight at 100°, abs. hexane/Et_2O 2:3) yielded from the faster eluting fraction pure

[16]) Under optimized reaction conditions only the desired (±)-*cis*-hydroxy ester was obtained. Less controlled reductions yielded mixture of the *cis*- and *trans*-ester in various ratios which had to be separated chromatographically (SiO_2, hexane/Et_2O 5:4).

[17]) The resolution of the (+)-enantiomer with brucine according to literature procedures [3] as well as several attempts with other chiral selectors were not reproducible in our hands. Therefore, the (+)-isomer was prepared by yeast reduction.

[18]) A full account on both the resolution and enantioselective synthesis of the precursors, the determination of the enantiomeric excesses (ee), and the absolute configurations of 3-substituted *cis*- and *trans*-2,4-dioxa-3λ^5-phosphabicyclo[4.4.0]decan-3-ones as well as on the difficulties encountered in this context will be published elsewhere [17]. The most significant results of that topic have been presented at the '1st Electronic Conference on Synthetic Organic Chemistry' (ECSOC-1), 1–30 September 1997; http://unibas.ch/mdpi/ecsoc/a0034/a0034.htm.

[19]) The reagent was prepared according to [18]. It consisted of $Cl_2P(O)ODnp$ (*ca.* 60%) (^{31}P-NMR ($CDCl_3$): δ 5.08) and $ClP(O)(ODnp)_2$ (*ca.* 25%) (^{31}P-NMR: δ 6.46).

axial epimer (±)-**4a** (89 mg, 30%), and from the slower eluting one the equatorial epimer (±)-**4b** (76 mg, 26%), both as slightly yellowish oils[20]).

(±)-**4a**: R_f (AcOEt) 0.64, R_f (*RP-188*, CH_2Cl_2) 0.56. IR ($CHCl_3$): 3005*w*, 2942*w*, 2861*w*, 1610*m*, 1539*s*, 1485*w*, 1348*s*, 1315*m*, 1267*m*, 1154*w*, 1130*w*, 1111*w*, 1090*m*, 1065*m*, 1005*s*, 984*m*, 958*w*, 933*m*, 901*m*, 833*w*, 620*w*. ^{1}H-NMR (300 MHz, $CDCl_3$): 1.25–1.45 (*m*, 1 H); 1.52–1.72 (*m*, 4 H); 1.80–2.09 (*m*, 4 H, incl. H_{eq}–C(10))[6]); 4.23 (*ddd*, ^2J = 11.2, ^3J(5eq,P) = 25.5, ^3J(5eq,6) = 1.1, H_{eq}–C(5)); 4.71 (*dd*, ^2J = 11.2, ^3J(5ax,6) = 2.4, ^3J(5ax,P) ≈ 0, H_{ax}–C(5)); 5.02 (br. *s*, $w_{1/2}$ ≈ 10, H–C(1)); 8.13 (br. *d*, ^3J(6′,5′) = 9.2, H–C(6′)); 8.47 (*dd*, ^3J(5′,6′) = 9.2, ^4J(5′,3′) = 2.7, H–C(5′)); 8.82 (*dd*, ^4J(3′,5′) = 2.7, ^5J(3′,6′) = 1, H–C(3′)). ^{13}C-NMR (75.4 MHz, $CDCl_3$): 18.6 (C(9)); 23.0 (C(8)); 24.5 (C(7)); 31.2 (*d*, ^3J(10,P) = 9.3, C(10)); 36.1 (*d*, ^3J(6,P) = 5.4, C(6)); 74.8 (*d*, ^2J(5,P) = 7.3, C(5)); 81.0 (*d*, ^2J(1,P) = 7.6, C(1)); 121.6 (C(3′)); 122.8 (C(6′)); 129.1 (C(5′)); 140.1 (*d*, ^3J(2′,P) = 8.9, C(2′)); 143.3 (C(4′)); 148.1 (*d*, ^2J(1′,P) = 5.0, C(1′)). ^{31}P-NMR (121.4 MHz, $CDCl_3$, ^{1}H-coupled): −15.0 (*dd*, ^3J(P,H_{eq}–C(5)) = 25.5, ^4J(P,H_{eq}–C(10)) = 4.5)[6]). ^{31}P{^{1}H}-NMR (242.9 MHz, MeCN (D_2O/0.2M *Tris* (pH 7.8) 11:44:45): −12.30. CI-MS (NH_3): 376 (100, $[M + NH_4]^+$), 359 (1.5, $[M + H]^+$), 210 (21).

(±)-**4b**: R_f (AcOEt) 0.44, R_f (*RP-188*, CH_2Cl_2) 0.30. IR ($CHCl_3$): 2927*m*, 2854*m*, 1609*m*, 1542*s*, 1485*w*, 1469*w*, 1450*w*, 1348*s*, 1306*m*, 1089*m*, 1053*s*, 1003*s*, 980*m*, 934*m*, 901*w*, 862*w*, 834*w*, 821*w*, 623*w*. ^{1}H-NMR (600 MHz, 323 K[21]), $CDCl_3$): 1.30–1.90 (*m*, 7 H); 2.07–2.17 (*m*, 1 H); 2.33 (*m*, $w_{1/2}$ ≈ 20, *X* of *ABX-P*, H–C(6)); 4.45, 4.52 (*AB* of *ABX-P*, ^2J ≈ ^3J(5,P) ≈ 11.5, ^3J(5,6) ≈ 7, 4.5, CH_2(5)); 4.84 (*ddt*, ^3J(1,P) = 11.8, ^3J(1,6) = 8, ^3J(1,10ax) ≈ ^3J(1,10eq) ≈ 4, H–C(1)); 7.95 (*dd*, ^3J(6′,5′) = 9.2, ^5J(3′,6′) = 1.2, H–C(6′)); 8.42 (*dd*, ^3J(5′,6′) = 9.2, ^4J(5′,3′) = 2.8, H–C(5′)); 8.78 (*dd*, ^4J(3′,5′) = 2.8, ^5J(3′,6′) = 1.2, H–C(3′)). ^{13}C-NMR (75.4 MHz, 323 K[21]), $CDCl_3$): 21.7 (C(9)); 22.4 (C(8)); 25.0 (C(7)); 29.5 (*d*, ^3J(10,P) = 2.9, C(10)); 35.7 (*d*, ^3J(6,P) = 7.1, C(6)); 72.2 (*d*, ^2J(5,P) = 6.7, C(5)); 82.5 (*d*, ^2J(1,P) = 7.1, C(1)); 121.2 (C(3′)); 123.9 (C(6′)); 128.6 (C(5′)); 140.9 (*d*, ^3J(2′,P) = 8.9, C(2′)); 143.7 (C(4′)); 148.1 (*d*, ^2J(1′,P) = 4.2, C(1′)). ^{31}P-NMR (121.4 MHz, ^{1}H-coupled, $CDCl_3$): −14.2 (*q*, ^3J(P,H–C(1)) ≈ ^3J(P,H_{ax}–C(5)) ≈ ^3J(P,H_{eq}–C(5)) ≈ 11.5). ^{31}P{^{1}H}-NMR (242.9 MHz, MeCN/D_2O/0.2M *Tris* (pH 7.8) 11:44:45): −11.43. CI-MS (2-methylpropane): 717 (100, $[2M + H]^+$), 367 (37, [pyrophosphate + H]$^+$), 359 (65, $[M + H]^+$), 301 (49)[22]).

4. *(+)-(1S,3S$_P$,6S)-and (−)-(1S,3R$_P$,6S)-3-(2,4-Dinitrophenoxy)-2,4-dioxa-3λ^5-phosphabicyclo[4.4.0]-decan-3-ones* ((±)-**4a** and (−)-**4b**, resp.). As described for (±)-**4a** and (±)-**4b**, starting from (+)-**3**. Final purification by prep. TLC gave (+)-**4a** and (−)-**4b**, both as slightly yellowish oils. (+)-**4a**: $[\alpha]_D^{20}$ = + 32.7 (*c* = 0.37, $CHCl_3$). (−)-**4b**: $[\alpha]_D^{20}$ = − 4.6 (*c* = 0.52, $CHCl_3$)[18])[20]). IR, ^{1}H-, ^{13}C-, and ^{31}P-NMR, and CI-MS: identical to those of (±)-**4a** and (±)-**4b**, resp.

5. *(−)-(1R,3R$_P$,6R)- and (+)-(1R,3S$_P$,6R)-3-(2,4-Dinitrophenoxy)-2,4-dioxa-3λ^5-phosphabicyclo[4.4.0]-decan-3-ones* ((−)-**4a** and (+)-**4b**). Analogously, starting from (−)-**3**, we obtained, after TLC purification, (−)-**4a** and (+)-**4b** as slightly yellowish oils. (−)-**4a**: $[\alpha]_D^{20}$ = − 22.5 (*c* = 0.28, $CHCl_3$). (+)-**4b**: $[\alpha]_D^{20}$ = + 3.1 (*c* = 0.42, $CHCl_3$)[18])[20]). IR, ^{1}H-, ^{13}C-, and ^{31}P-NMR, and CI-MS: identical to those of (±)-**4a** and (±)-**4b**, resp.

6. *N-[(Benzyloxy)carbonyl]-*O*-(*cis*-3-oxo-2,4-dioxa-3λ^5-phosphabicyclo[4.4.0]dec-3-yl)-*L*-serine Methyl Ester* (**6′a**/**6′b**; both mixture of diastereoisomers[2]). To a soln. of $POCl_3$ (130 µl, 1.43 mmol) and pyridine (122 µl, 1.51 mmol) in abs. Et_2O (3 ml), prepared at 0° in a glove-box under N_2, a soln. containing *N*-[(benzyloxy)carbonyl]-L-serine methyl ester (384 mg, 1.51 mmol) was added (*in situ* formation of **5**). After stirring for 2 h at 0°, (±)-**3** (186 mg, 1.43 mmol) and pyridine (231 ml, 2.86 mmol) in abs. Et_2O (1.5 ml) were added. The mixture was stirred for 12 h at 20° and then evaporated. CC (silica gel, Et_2O/MeOH 50:1) of the crude product (700 mg) yielded the faster eluting axial epimer **6′a** (160 mg, 26%) followed by the equatorial epimer **6′b** (141 mg, 23%), both as a mixture (*ca.* 1:1) of diastereoisomers[2]).

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[20]) The equatorial epimers are labile compounds; their yield and purity strongly depended on the applied chromatographic conditions and the workup procedures. Prior to any further experiment, (±)-, (+)-, and (−)-**4b** were purified by prep. TLC on reversed-phase silica gel (*RP-188* F_{254S} on glass plates (*Merck*), CH_2Cl_2).

[21]) Due to coalescence phenomena, badly resolved spectra were observed at T < 300 K. Moreover, high concentrations (*ca.* 20 mg/0.5 ml) gave rise to broad lines at T > 320 K, whereas low concentrations (*ca.* 3 mg/0.5 ml) yielded well-resolved spectra at T > 320 K.

[22]) The MS behaviour of **4a** was significantly different from that of **4b**. Under various ionization conditions (CI (NH_3), ESI, *etc.*), the equatorial epimers **4b** always exhibited additional peaks due to $[2M + H]^+$ and the corresponding pyrophosphate. The latter was the 100% peak when NH_3 was used as reactant gas.

6'a ((2*S*,1'*S*,3'R_p,6'*S*)/(2*S*,1'*R*,3'S_p,6'*R*)): ^{13}C-NMR (75.4 MHz, $CDCl_3$): 73.1, 73.3 (*d*, ^2J(5',P) = 5.7, 5.8, C(5')); 78.9, 79.0 (*d*, ^2J(1',P) = 8.5, 7.4, C(1')). ^{31}P-NMR (121.4 MHz, $CDCl_3$, ^{1}H-coupled): −7.5 (*d*, ^3J(P,H_{eq}−C(5')) = 24). ^{31}P{^{1}H}-NMR (242.9 MHz, MeCN/D_2O/0.2M *Tris* (pH 7.8) 11:44:45): −5.67.

6'b ((2*S*,1'*S*,3'S_p,6'*S*)/(2*S*,1'*R*,3'R_p,6'*R*)): ^{13}C-NMR (75.4 MHz, $CDCl_3$): 71.5, 71.6 (*d*, ^2J(5',P) = 3.3, 4.8, C(5')). ^{31}P-NMR (121.4 MHz, $CDCl_3$, ^{1}H-coupled): −4.9 (*m*, $w_{1/2} \approx 35$). ^{31}P{^{1}H}-NMR (242.9 MHz, MeCN/D_2O/0.2M *Tris* (pH 7.8) 11:44:45): −4.02, −4.20.

The enantiomerically pure diastereoisomers (−)-**6a** and (+)-**6b** were prepared analogously, starting from (−)-**3**.

*(−)-(2S,1'R,3'S$_P$,6'R)-*N*-[(Benzyloxy)carbonyl]-*O*-(3-oxo-2,4-dioxa-3λ^5-phosphabicyclo[4.4.0]dec-3-yl)-*L*-serine Methyl Ester* ((−)-**6a**): Colourless viscous oil. R_f (AcOEt) 0.48. $[\alpha]_D^{20} = -21.3$ (*c* = 1.65, EtOH), $[\alpha]_D^{20} \approx 0$ (*c* = 1.21, $CHCl_3$). IR ($CHCl_3$): 3430*w*, 3000*w*, 2940*m*, 2860*w*, 1723*s*, 1513*m*, 1450*w*, 1436*w*, 1342*w*, 1295*s*, 1155*w*, 1111*w*, 1083*m*, 1064*m*, 1002*s*, 978*m*, 952*m*, 926*w*, 914*w*, 620*w*. ^{1}H-NMR (300 MHz, $CDCl_3$): 1.28 (*m*, *q*-like, $w_{1/2} \approx 30$, 2 H); 1.42–1.75 (*m*, 4 H); 1.75–2.00 (*m*, *d*- and *t*-like, 3 H); 3.80 (*s*, MeO); 4.01 (*dd*, ^2J = 11.2, ^3J(5'eq,P) = 24, ^3J(5'eq,6') ≈ 0, H_{eq}−C(5')); 4.27 (*dd*, ^2J = 11.2, ^3J(5'ax,6') = 1.1, ^3J(5'ax,P) ≈ 0, H_{ax}−C(5')); 4.35–4.52 (*AB* of *ABX-P*, not resolved, CH_2(3)); 4.61 (br, *s*, $w_{1/2} \approx 12$, H−C(1'), H−C(2)); 5.13 (*s*, PhCH_2); 5.83 (*d*, ^3J(NH,2) = 7.7, NH−C(2)); 7.31–7.37 (*m*, 5 arom. H). ^{13}C-NMR (75.4 MHz, $CDCl_3$): 18.6 (C(9')); 22.9 (C(8')); 24.6 (C(7')); 31.3 (*d*, ^3J(10',P) = 8.8, C(10')); 36.1 (*d*, ^3J(6',P) = 5.6, C(6')); 52.8 (MeO); 54.4 (*d*, ^3J(2,P) = 6.7, C(2)); 66.6 (*d*, ^2J(3,P) = 5.2, C(3)); 67.1 (PhCH_2); 73.3 (*d*, ^2J(5',P) = 7.1, C(5')); 79.0 (*d*, ^2J(1',P) = 6.9, C(1')); 128.1, 128.2, 128.4 (arom. CH); 136.0 (arom. C); 155.7 (OCON); 169.5 (C(1)). ^{31}P-NMR (121.4 MHz, $CDCl_3$ ^{1}H-coupled): −7.5 (*d*, ^3J(P,H_{eq}−C(5')) = 24). ^{31}P{^{1}H}-NMR (242.9 MHz, MeCN/D_2O/0.2M *Tris* (pH 7.8) 11:44:45): −5.67. ESI-MS (MeOH/$CHCl_3$/NaI): 450 (100, $[M + Na]^+$).

*(+)-(2S,1'R,3'R$_P$,6'R)-*N*-[(Benzyloxy)carbonyl]-*O*-(3-oxo-2,4-dioxa-3λ^5-phosphabicyclo[4.4.0]dec-3-yl)-*L*-serine Methyl Ester* ((+)-**6b**): Colourless viscous oil. R_f (AcOEt) 0.23. $[\alpha]_D^{20} = +9.8$ (*c* = 0.97, $CHCl_3$). IR ($CHCl_3$): 3425*w*, 3000*w*, 2940*m*, 2860*w*, 1722*s*, 1511*m*, 1452*w*, 1437*w*, 1381*w*, 1342*m*, 1152*w*, 1085*m*, 1060*s*, 1000*s*, 979*m*, 961*w*, 944*m*, 914*w*, 620*w*. ^{1}H-NMR (300 MHz, $CDCl_3$): 1.18–2.04 (*m*, 9 H); 3.78 (*s*, MeO); 4.12 (*ddd*, ^2J = 11.3, ^3J(5'eq,P) = 18, ^3J(5'eq,6') = 3.0, H_{eq}−C(5')); 4.37–4.62 (*m* and *ABX* of *ABX-P*, not resolved, H_{ax}−C(5'), CH_2(3), H−C(2)); 4.80 (*m*, *quint.*-like, $w_{1/2} \approx 12$, H−C(1')); 5.13 (*s*, PhCH_2); 5.82 (*d*, ^3J(NH,2) = 7.8, NH−C(2)); 7.31–7.37 (*m*, 5 arom. H). ^{13}C-NMR (75.4 MHz, $CDCl_3$): 19.9 (C(9')); 23.8 (C(8')); 24.0 (C(7')); 30.5 (*d*, ^3J(10',P) = 7.0, C(10')); 35.9 (*d*, ^3J(6',P) = 5.0, C(6')); 52.8 (MeO); 54.3 (*d*, ^3J(2,P) = 7.3, C(2)); 67.1 (PhCH_2); 67.9 (*d*, ^2J(3,P) = 5.7, C(3)); 71.6 (*d*, ^2J(5',P) = 5.4, C(5')); 78.3 (*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, $CDCl_3$, ^{1}H-coupled): −4.9 (*m*, $w_{1/2} \approx 35$). ^{31}P{^{1}H}-NMR (242.9 MHz, MeCN/D_2O/0.2M *Tris* (pH 7.8) 11:44:45): −4.02. ESI-MS (MeOH/$CHCl_3$/NaI): 450 (100, $[M + Na]^+$).

7. *^{31}P-NMR Experiments. Sample Preparation.* Soln. A: the inhibitor (0.6 mg, 1.65 μmol) was dissolved in abs. MeCN (60 μl) and thermostatted at 300 ± 1 K. Soln. B: δ-chymotrypsin (40 mg, 1.6 μmol) was dissolved in D_2O (240 μl) and 0.2M *Tris* buffer (250 μl, pH 7.8) in an NMR tube (5 mm i.d.) and thermostatted in the NMR spectrometer at 300 ± 1 K. After homogenizing the magnetic field, the sample was removed, soln. A quickly added by use of a micropipette and thoroughly mixed, the sample reinserted, and the run started after reshimming. The total time required from mixing of the components to the start of the runs was *ca.* 10 min.

^{31}P-NMR Parameters: *Bruker AM-600*, 242.9 MHz, temp. 300 ± 1 K; δ (^{31}P) in ppm rel. to 85% H_3PO_4 (= 0 ppm) as external reference; pulse width 8 μs (45°); relaxation delay 1 s; acquisition time 0.72 s, spectral width 17000 Hz (70 ppm); line broadening factor 2.0 Hz.

Time Program: At the beginning of inhibition, two spectra of 1000 transients were recorded (*ca.* 30 min each) and the FID's (*f*ree *i*nduction *d*ecays) saved; then spectra with 2000 transients were recorded (*ca.* 1 h each). For better signal to noise ratio, several FID's consecutively accumulated in the time period of interest were added and the desired spectra obtained by *Fourier* transformation.

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