Covalent-Bond Formation in the Course of the Inhibition of δ-Chymotrypsin with *trans*-Decalin-Type Organophosphates: ^{31}P-NMR Evidence

by **Markus J. Stöckli** and **Peter Rüedi***

Organisch-chemisches Institut der Universität Zürich, Winterthurerstrasse 190, CH-8057 Zürich

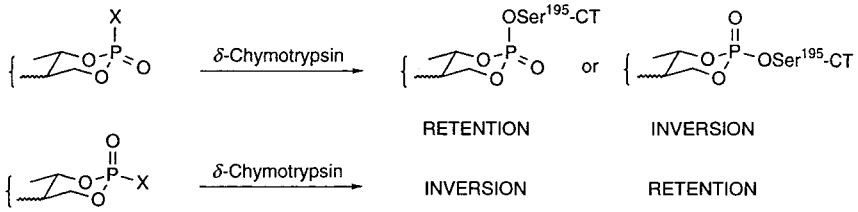
Earlier investigations have shown that the irreversible inhibition of δ-chymotrypsin with the axially substituted *trans*-3-(2,4-dinitrophenoxy)-2,4-dioxa-3λ^5-phosphabicyclo[4.4.0]decan-3-one (= 2-(2,4-dinitrophenoxy)hexahydro-4*H*-1,3,2-benzodioxaphosphorin 2-oxide) proceeds under inversion of the configuration at the P-atom. Since this assignment is based on the comparison of the respective chemical shifts with model compounds, the covalent nature of the binding interaction between enzyme and inhibitor was formulated in analogy. To prove this assumption, inhibition experiments were performed with the deuterated inhibitor (±)-*trans*-3-(2,4-dinitrophenoxy)-2,4-dioxa-3λ^5-phospha(1,5,5-$^2H_3$)bicyclo[4.4.0]decan-3-one ((±)-**6a**). ^{31}P{^{2}H}-NMR-Spectroscopic monitoring of the reaction of stoichiometric amounts of the enzyme with (±)-**6a** at pH 7.8 yielded the diastereoisomeric adducts **9** (– 3.88 ppm) and **9′** (– 3.96 ppm). Comparing the ^{31}P chemical shifts of the corresponding deuterated covalent phosphoserine model compounds **8a/8a′** (– 6.70 ppm, axial) and **8b/8b′** (– 4.11/– 4.13 ppm, equatorial) confirmed the inversion of the configuration at the P-atom. ^{1}H-Correlated ^{31}P{^{2}H}-NMR spectra revealed a cross peak of the Ser^{195}-H_2 (4.45 ppm) with the P-atom of the inhibitor at – 3.88/– 3.96 ppm, thus establishing the covalent nature of the Ser^{195}–O–P bond.

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 stereochemical pathways of the inhibition reaction by ^{31}P-NMR spectroscopy (for the general concept, see *Scheme 1*), we reported on the enantiomeric *cis*- and *trans*-3-(2,4-dinitrophenoxy)-2,4-dioxa-3λ^5-phosphabicyclo[4.4.0]decan-3-ones (= hexahydro-4*H*-1,3,2-benzodioxaphosphorin 2-oxides) [1][2][1]). These configurationally and conformationally locked *cis*- and *trans*-decalin congeners follow different kinetic and stereochemical courses in the inhibition of δ-chymotrypsin. The four *trans*-isomers and the (–)-enantiomer of the equatorially *P*-substituted *cis*-compound (1*S*,3R_p,6*S*)-configuration) are irreversible inhibitors, whereas the three other *cis*-isomers inhibited the enzyme reversibly. Based on the comparison of the respective ^{31}P-chemical shifts with those of covalent phosphoserine model compounds, it was demonstrated that the axially substituted epimers of the *trans*-series react *via* neat inversion, their equatorially substituted counterparts show both inversion and retention [1], whereas the *cis*-compound exhibits neat retention [2] of the configuration at the P-atom.

Since the existence of covalent Ser^{195}–O–P bonds between the enzyme and the inhibitors has been concluded from the chemical-shift analogy, we considered this statement most probably but not fully unambiguous. Therefore, we decided to confirm

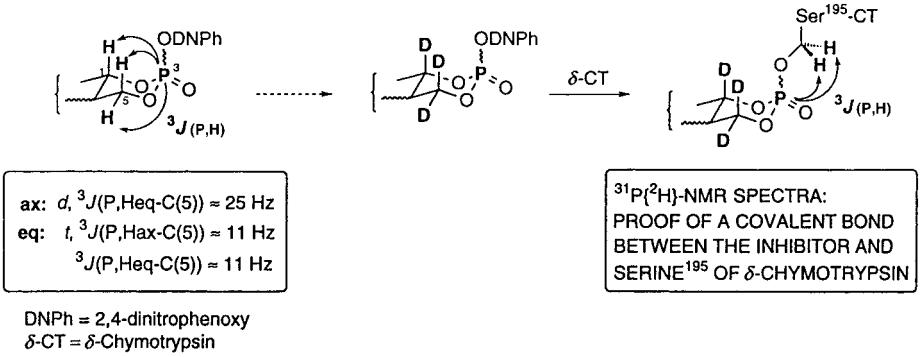
[1]) For a full account on the background of the project, its context, and pertinent references, see [1].

Scheme 1



our assumption by the concept outlined in *Scheme 2:* In the case of a covalent bond, a vicinal coupling (^3J) between the P-atom of the inhibitor and the Ser^{195}-H_2 of the enzyme exists, and it can be evidenced in the ^{1}H-coupled ^{31}P-NMR spectra. However, due to the splitting caused *a priori* by H–C(1) and CH_2(5) of the oxaphosphadecaline[2]), the crucial ^3J(P,H) coupling can be detected reliably only when the coupling with the H-atoms of the inhibitor is supressed. This is accomplished most simply by replacing them with deuterium. However, to obtain the reliable information, ^{2}H-decoupled, ^{1}H-coupled ^{31}P-NMR spectra have to be recorded, as ^{2}H also couples with the ^{31}P-atom[3])[4]). Compared to the results documented in [1] and [2], these prerequisites are unfavorable with respect to the expected intensities of the resonances.

Scheme 2



---

[2]) The magnitude of the vicinal ^3J(P,H) is the key argument in assigning the conformation of the heterocyclic ring and, as a consequence, the configuration at the P-atom (*Scheme 2*) (*cf.* [1][2] and refs. cit. therein).

[3]) This holds at least for the 1D spectra. Sophisticated 2D experiments would allow us to avoid that particular problem, but as the δ values of H–C(1) and CH_2(5) in the *protio*-isomer of (±)-**6a** are similar to those of the Ser^{195}-H_2, recording ^{31}P{^{2}H}-spectra was considered to be the most straightforward approach. In general, coupling with nuclei of different quantum numbers causes complex splitting patterns that render the interpretation difficult. Although ^3J(P,D) is usually negligibly small and would only contribute to line broadening, it becomes significant in the present case ($^3J(P,H_{eq}-C(5)) \approx 25$ Hz and the expected $^3J(P,^2H_{eq}-C(5)) \approx 3.5$ Hz).

[4]) Since the ^{2}H-channel is usually the lock signal, this requirement is not trivial.

Coupling of ^{31}P with ^{1}H and quadrupole relaxation significantly decrease the intensity of the signals. Moreover, with racemic inhibitors, two signals will be generated as both diastereoisomeric adducts are formed [1].

In spite of these disadvantages, we started with the most simple setup as the key-experiment. According to our experience, the axially *P*-substituted (±)-*trans*-3-(2,4-dinitrophenoxy)-2,4-dioxa-3λ^5-phospha(1,5,5-$^2H_3$)bicyclo[4.4.0]decan-3-one ((±)-**6a**) was chosen as inhibitor. In analogy to the *protio*-isomer [1], the compound was considered stable and to react with inversion of the configuration at the P-atom.

Results and Discussion. – *Synthesis and Characterization of the Deuterated Phosphadecalins.* – The *trans*-3-(2,4-dinitrophenoxy)-2,4-dioxa-3λ^5-phospha(1,5,5-$^2H_3$)bicyclo[4.4.0]decan-3-ones ((±)-**6a** and (±)-**6b**) have been obtained by reduction of ethyl 2-oxocyclohexanecarboxylate (**1**) with $NaBD_4$, chromatographic separation of the resulting *cis*- and *trans*-2-hydroxy (2-$^2H_1$)carboxylates ((±)-**2** and (±)-**3**, resp.), reduction with $LiAlD_4$ to the corresponding *cis*- and *trans*-2-(hydroxy($^2H_2$)methyl)(1-$^2H_1$)cyclohexan-1-ols ((±)-**4** and (±)-**5**, resp.). Reaction of (±)-**4** with 2,4-dinitrophenyl phosphorodichloridate and chromatographic separation of the resulting mixture (axial/equatorial *ca.* 1:1) yielded the pure epimers (±)-**6a** and (±)-**6b** (*Scheme 3*). The axially and equatorially substituted phosphoserine model compounds **8a**/**8a′** and **8b/8b′** were prepared from (±)-**4** and *N*-[(benzyloxy)carbonyl]-*O*-(dichlorophosphinyl)-L-serine methyl ester (**7**; generated *in situ*) and obtained as a mixture of diastereoisomers (*ca.* 1:1) after chromatographic separation (*Scheme 3*).

The spectroscopic data of the deuterated dioxaphosphadecalins (±)-**6a**, (±)-**6b**, **8a/8a′**, and **8b/8b′** fully confirmed their structures (see *Exper. Part*). Significant ^1H- and ^{13}C-NMR characteristics are both the lack of the *ABX*-P-system in the heterocyclic moiety and, due to $^1J(C,D)$ and $^2J(C,P)$, the complex *multiplets* for C(1) and C(5) in (±)-**6a**/(±)-**6b**, and of C(1′) and C(5′) in **8a/8a′** and **8b/8b′**, which hardly exceed the noise. Compared to the *protio*-isomers [1], $\delta(^{31}P)$ remained almost unchanged, and the axially substituted epimers again resonated at higher field with respect to the equatorially substituted counterparts. The decisive spectral feature is provided by the $^{31}P\{^2H\}$-NMR spectra (*Figs. 1* and *2*) that exhibit a *triplet* at −6.70 ppm ($(^3J(P,CH_2(3)) = 7.0$ Hz)[5]) for **8a/8a′** and two *triplets* at −4.11/−4.13 ppm ($(^3J(P,CH_2(3)) = 6.5$ Hz)[5]) for **8b/8b′**. The ^1H-correlated $^{31}P\{^2H\}$-NMR spectra clearly showed the respective cross peaks with $H_a-C(3)$ and $H_b-C(3)$ of the serine moiety at 4.38/4.63 ppm (**8a/8a′**) and at 4.37/4.50 ppm (**8b/8b′**), and indicated also a $^4J(P,H-C(2))$ coupling in **8b/8b′**.

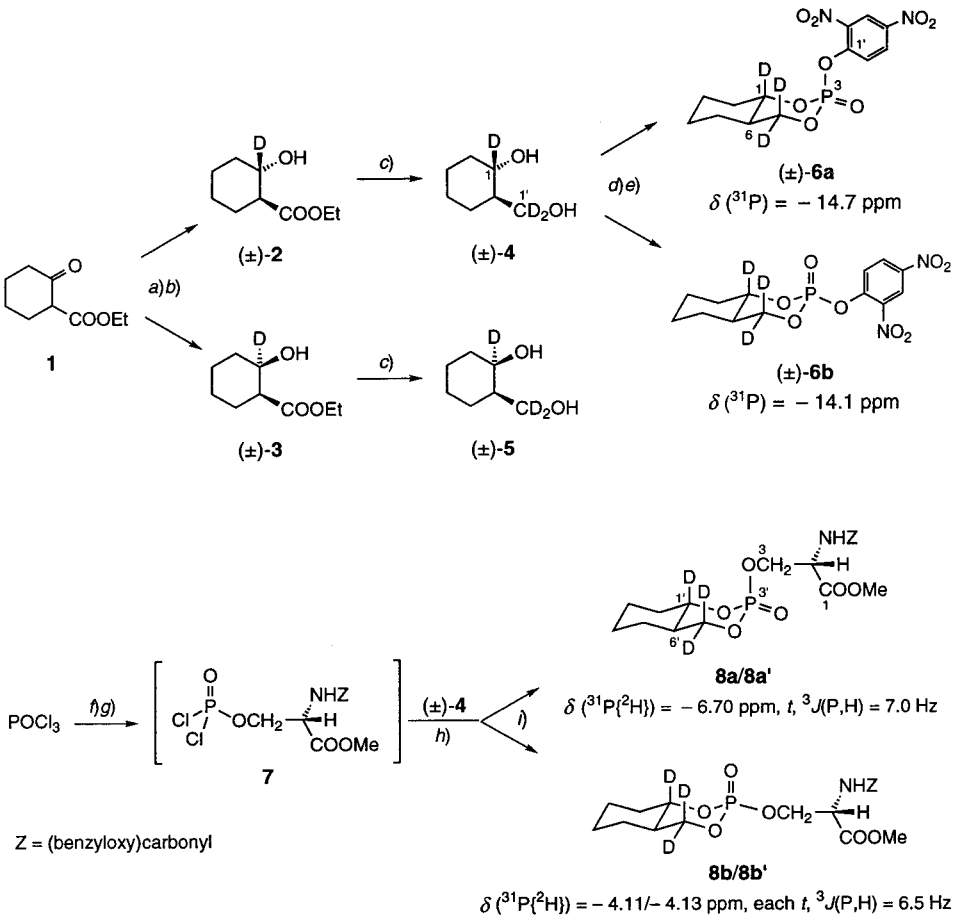
^{31}P-NMR Investigations. Similar to the preceding setups [1][2], the experiments were designed so that the enzyme and the inhibitor (±)-**6a** reacted in stoichiometric amounts (*ca.* 2 μmol in *Tris* buffer at pH 7.8 (27°)). Due to the expected low sensitivity, the progression of the inhibition reaction was not spectroscopically followed as described in [1], and the ^{31}P-NMR spectra were recorded only after complete inhibition had taken place.

The result is shown in *Fig. 3:* the 1D $^{31}P\{^2H\}$-NMR spectrum strongly resembles the $^{31}P\{^1H\}$-NMR spectrum of the reaction of δ-chymotrypsin with the *protio*-isomer of

---

[5] The signal is formally a *dd* as $H_a-C(3)$ and $H_b-C(3)$ are diastereotopic.

Scheme 3



a) $NaBD_4$, EtOH, $-10°$, 30 min. *b*) Chromatography (SiO_2; hexane/Et_2O 5:4). *c*) $LiAlD_4$, Et_2O, $0° \rightarrow$ reflux, 12 h. *d*) $Cl_2P(O)C_6H_3(NO_2)_2$, pyridine, $CHCl_3$, $0° \rightarrow$ r.t., 12 h. *e*) Chromatography (SiO_2; hexane/AcOEt 1:2). *f*) Pyridine, Et_2O, 0°. *g*) (+)-*N*-[(benzyloxy)carbonyl]-L-serine methyl ester, Et_2O, 0°, 2 h. *h*) Pyridine, Et_2O, r.t., 12 h. *i*) Chromatography (SiO_2; $CHCl_3$/AcOEt 1:1).

(±)-**6a** [1][6]). However, the signals are broadened and split to some extent, an observation that indicates a P,H coupling. Comparison of the ^{31}P chemical shifts with those of the model compounds **8a/8a′** (−6.70 ppm) and **8b/8b′** (−4.11/−4.13 ppm) shows that the resonances at −3.88 and −3.96 ppm should be attributed to the enzyme/inhibitor adducts **9** and **9′** in which the Ser^{195} moiety of δ-chymotrypsin occupies the

[6]) The intensive signal at −1.87 ppm is assigned to the hydrolysis product of (±)-**6a**. Its formation is explained by the combined mechanism of action of the serine hydrolases, which involves both general acid-base and covalent catalysis [3].

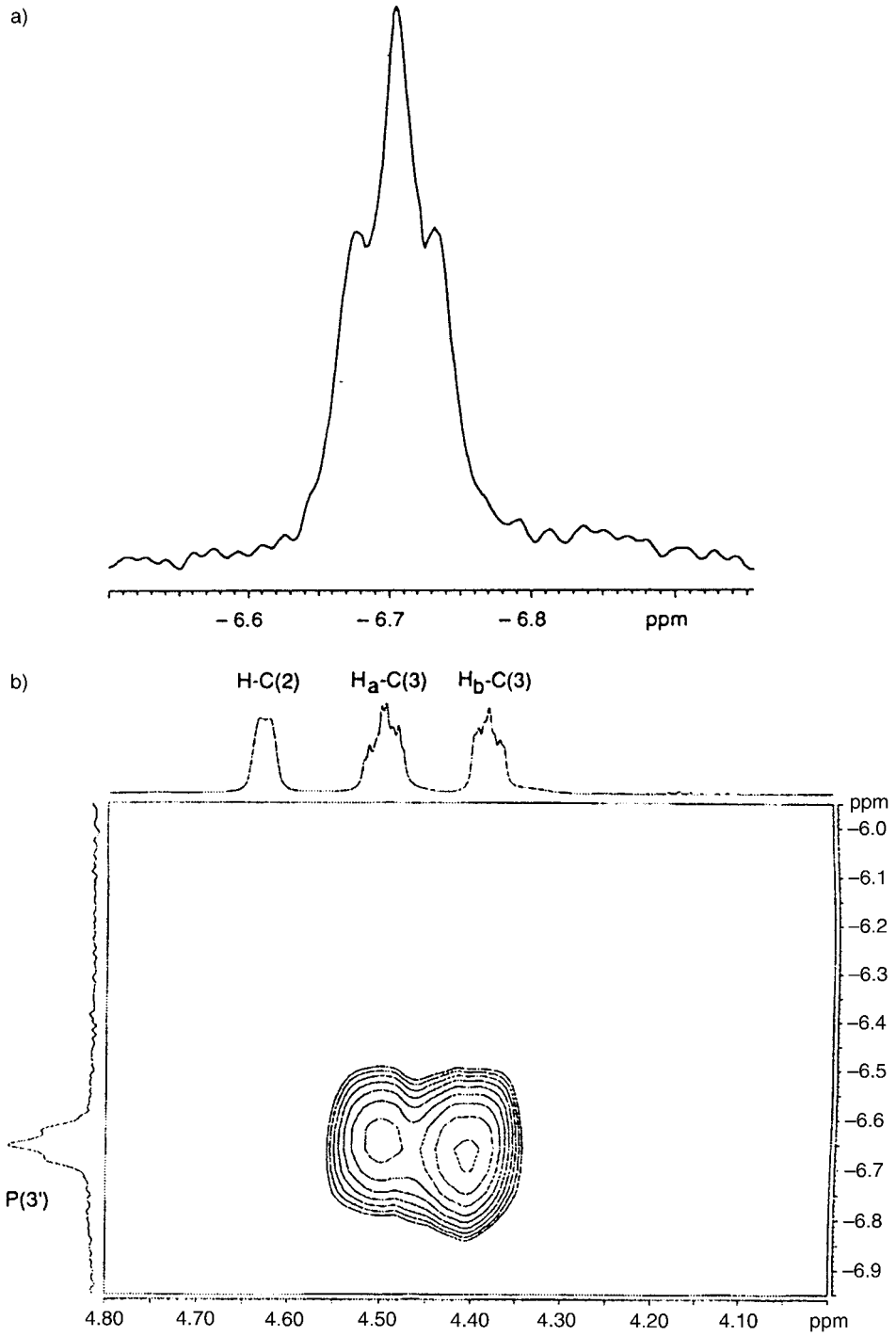


Fig. 1. *$^{31}P\{^{2}H\}$-NMR Spectra* (242.9 MHz, $CDCl_3$, 27°) *of the axially substituted model compound* **8a/8a′**. *a*) 1D spectrum; *b*) ^{1}H-correlated spectrum.

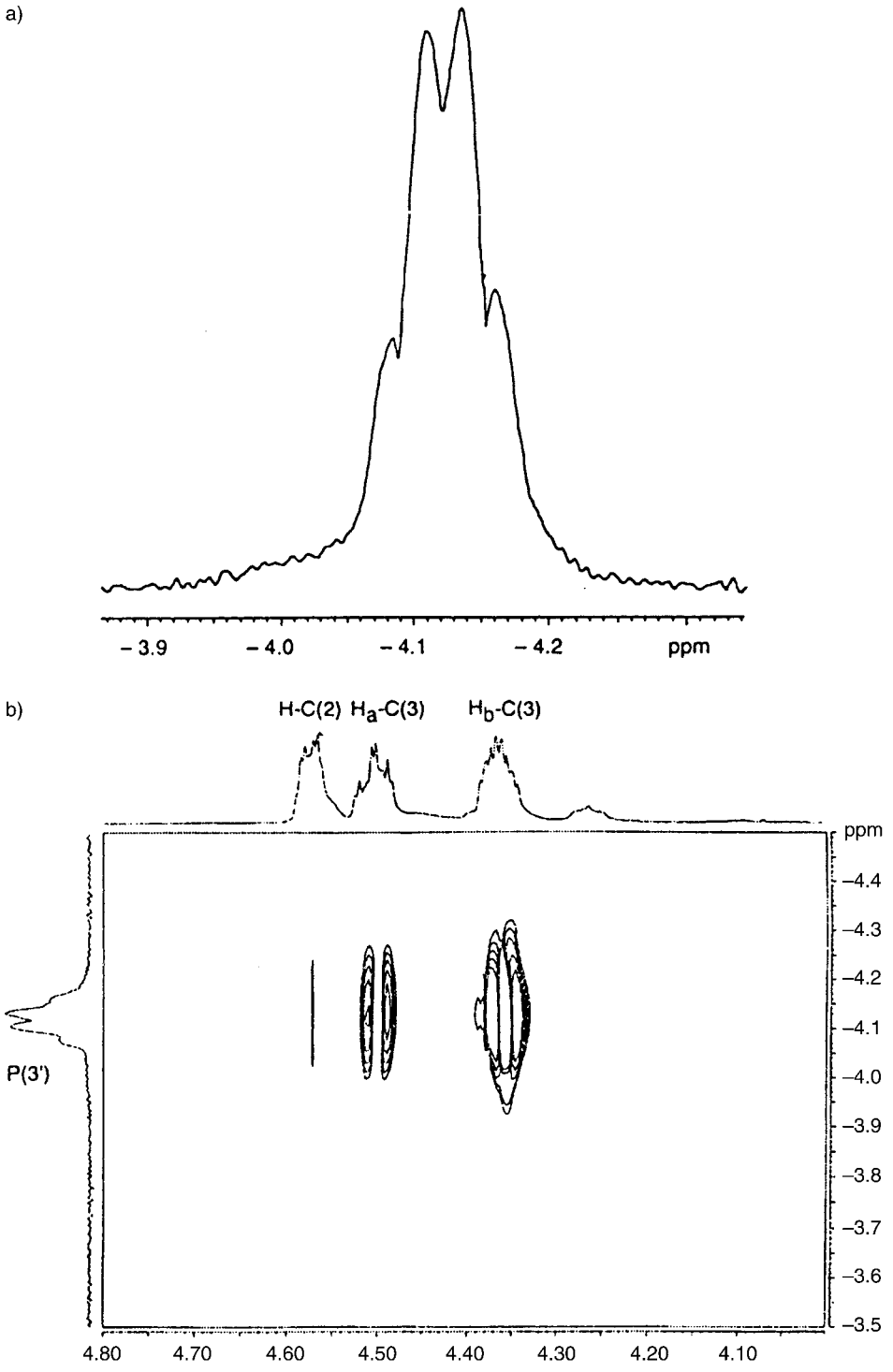


Fig. 2. *$^{31}P\{^{2}H\}$-NMR Spectra* (242.9 MHz, $CDCl_3$, 27°) *of the equatorially substituted model compound* **8b/8b′**. *a*) 1D Spectrum; *b*) ^{1}H-correlated spectrum.

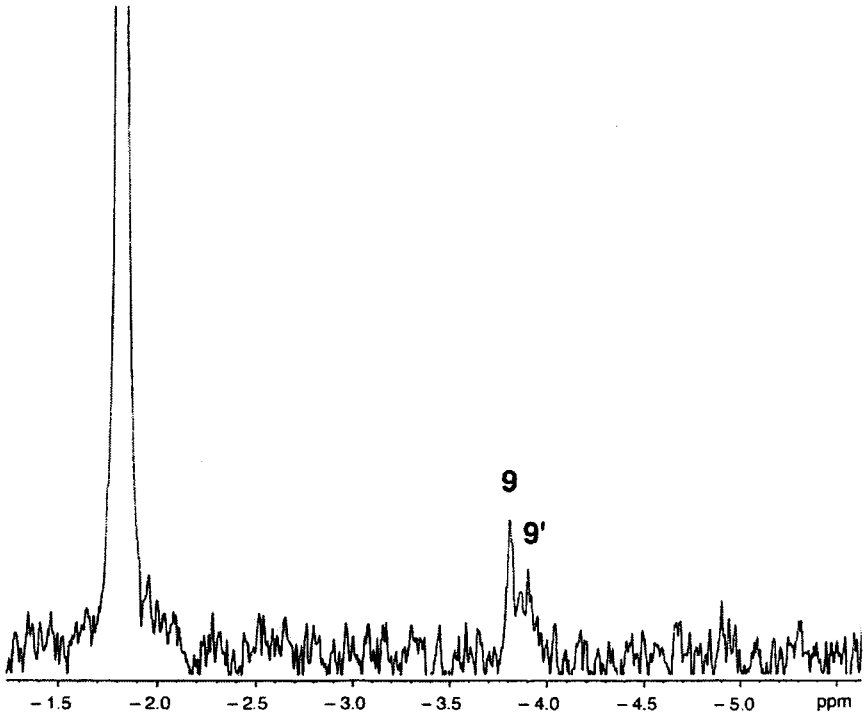
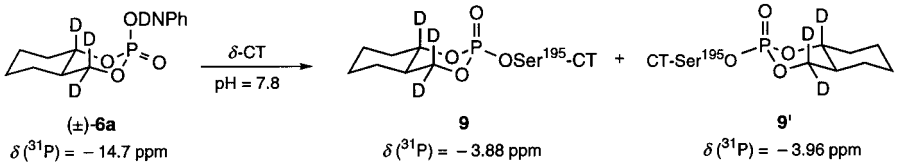


Fig. 3. *$^{31}P\{^{2}H\}$-NMR Spectrum* (242.9 MHz, 27°) *of the inhibition of δ-chymotrypsin with (±)-***6a**. Signal at – 1.87 ppm: hydrolyzed inhibitor; signals centered at 3.9 ppm: diastereoisomeric phosphoenzymes **9** (– 3.88 ppm) and **9′** (– 3.96 ppm). Solvent: 0.2M *Tris* buffer, pH 7.8 (45%), D_2O (44%), MeCN (11%).

Scheme 4



equatorial position at the P-atom[7]). Hence, it could be verified that the inhibition reaction followed a direct in-line displacement process [4] with retention of the configuration at the P-atom as discussed in detail in [1] (*Scheme 4*). Depending on the mathematical processing of the FID, *triplet* characters of the signals at – 3.88 and – 3.96 ppm can be suggested, but the low resolution does not allow a conclusive interpretation. The existence of a covalent bond is decisively demonstrated by the 2D ^{1}H-correlated $^{31}P\{^{2}H\}$-NMR spectrum that reveals a cross-peak of the P-atom of the inhibitor at – 3.88/ – 3.96 ppm with the ^{1}H resonances at *ca.* 4.45 ppm (*Fig. 4*).

---

7) The diastereoisomeric phosphoenzymes **9** and **9′** have been assigned in analogy to the findings in [1], where both the racemic and the enantiomerically pure *protio*-inhibitors were examined.

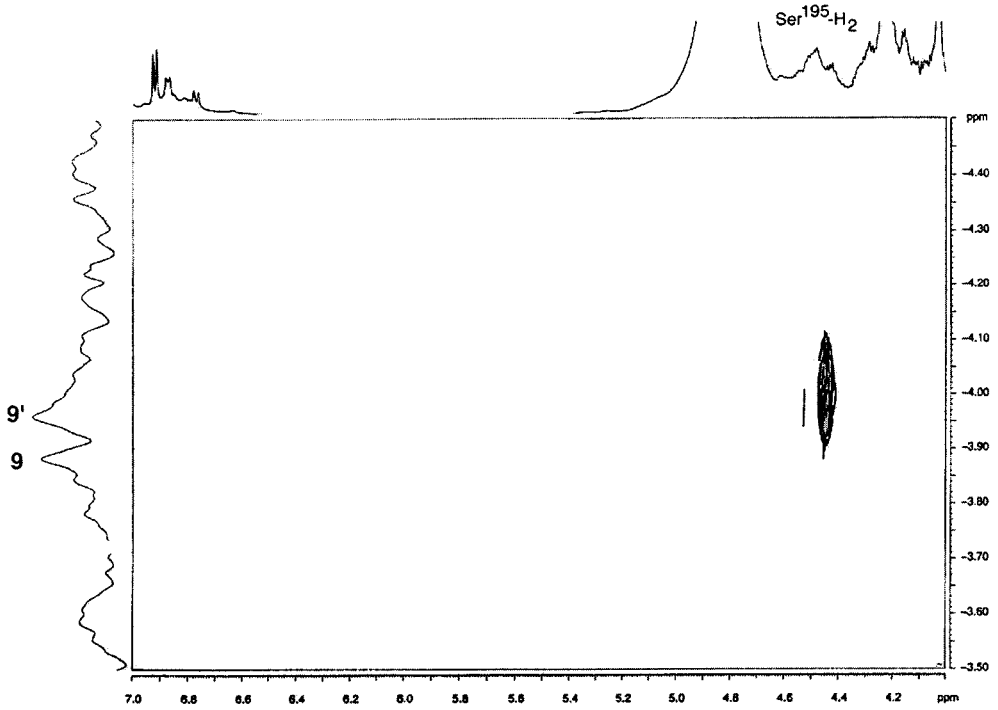


Fig. 4. *^{1}H-Correlated $^{31}P[^{2}H]$-NMR spectrum* (242.9 MHz, 27°) *of the inhibition of δ-chymotrypsin with (±)-***6a**. The cross-peak centered at 4.45 ppm (^{1}H, Ser^{195}-H_2) and −4.0 ppm (^{31}P) indicates the covalent $Ser^{195}-O-P$ bond. Solvent: 0.2M *Tris* buffer, pH 7.8 (45%), D_2O (44%), MeCN (11%).

According to the ^{1}H chemical shifts of the model compounds **8a/8a′** and **8b/8b′**, this signal group is allocated to the Ser^{195}-H_2.

This result proves that the irreversible inhibition of δ-chymotrypsin proceeds *via* covalent-bond formation between the nucleophilic $O-C(\beta)$ of the active-site serine^{195} residue in the catalytic triade ($Asp^{102}\cdots His^{57}\cdots Ser^{195}$) in the enzyme [5] and the P-atom of the inhibitor (±)-**6a**. The phosphorylated enzyme is a stable tetrahedral adduct and considered an analogue of the tetrahedral carbonyl addition intermediate or its transition state [6].

The authors are indebted to the *Swiss National Science Foundation* for the financial support and to the NMR team of our institute (*Martin Binder*, Dr. *Gudrun Hopp-Rentsch*, and *Nadja Walch*) for the skilful fulfillment of our weird desires.

Experimental Part

1. *General*. See [1]. In addition, column chromatography (CC) of the phosphadecalines on modified silica gel *60 HR* (0.015–0.040 mm, *Merck*, Art *774*): 200 g of silica gel were washed with 1M HCl, thoroughly neutralized with H_2O, and re-activated by stepwise washing with EtOH, hexane and dried at 120° (pH of an aqueous slurry 5.7). IR Spectra: *Perkin-Elmer Paragon 1000 PC* FT-IR spectrometer. NMR Spectra: *Bruker DRX-500* and *Bruker DRX-600* spectrometers; the spectral assignments in the oxaphosphadecalines are based on $^{1}H,^{1}H$-COSY-, $^{1}H,^{1}H$-NOESY-, $^{13}C/^{1}H$-HSQC- and HMBC spectra. GC/MS: *Hewlett-Packard HP-5980*

Series *II* (GC), *HP-5971 MSD* (mass-selective detector, 70 eV), column *HP-5*, 25 m × 0.2 mm, 0.33 μ; temp. program: 100° (2 min), 100°–240° (rate 20°/min), 240° (5 min).

2. *(±)*-cis- *and* trans-*Ethyl 2-Hydroxy(2-$^2H_1$)cyclohexanecarboxylate* ((±)-**2** and (±)-**3**, resp.). Reduction of ethyl *2-oxocyclohexanecarboxylate* (**1**; 16.6 g, 95.7 mmol) with $NaBD_4$ (1.40 g, 33.4 mmol, 0.35 equiv.) in EtOH (250 ml) for 3 h at 0°, workup and continuous extraction with Et_2O yielded the mixture (±)-**2**/(±)-**3** as a slightly greenish oil (15.62 g, 96%). CC (silica gel *60*; hexane/Et_2O 5:4) afforded pure (±)-**2** (*cis*-isomer; 8.23 g, 59%) and (±)-**3** (*trans*-isomer; 5.29 g, 37%).

Data of (±)-**2**: Colorless viscous oil. R_f (hexane/Et_2O 5:4) 0.26. GC: t_R 6.30 min. IR (Film): 3511*s*, 2979*s*, 2935*s*, 2855*s*, 2669*w*, 2154*w*, 2117*w*, 1894*w*, 1715*s*, 1447*s*, 1310*s*, 1187*s*, 989*s*, 873*m*, 785*m*, 665*m*. ^{1}H-NMR (300 MHz, $CDCl_3$): 4.16 (*q*, ^3J = 7.1, MeCH_2); 3.66 (*d*, ^4J = 1.1, OH); 2.47 (*dd*, ^3J = 11.0, ^3J = 4.0, H–C(1)); 1.96–1.80 (*m*, H_{eq}–C(3), H_{eq}–C(6)); 1.75–1.62 (*m*, H_{ax}–C(3), H_{eq}–C(4), H_{eq}–C(5), H_{ax}–C(6)); 1.51–1.37 (*m*, H_{eq}–C(4), H_{eq}–C(5)); 1.27 (*t*, ^3J = 7.1, *Me*CH_2). ^{13}C-NMR (75.4 MHz, $CDCl_3$): 175.8 (C=O); 66.3 (*t*, ^1J(C,D) = 22.5, C(2)); 60.6 (MeCH$_2$); 46.6 (C(1)); 31.7 (C(3)); 24.8 (C(5)); 24.0 (C(6)); 20.1 (C(4)); 14.2 (*Me*CH$_2$). EI-MS: 173 (2, M^+, $[C_9H_{15}DO_3]^+$), 155 (20, $[M-H_2O]^+$), 145 (60, $[M-C_2H_4]^+$), 128 (18), 116 (10), 110 (22), 101 (95), 82 (73), 73 (100).

Data of (±)-**3**: Colorless viscous oil. R_f (hexane/Et_2O 5:4) 0.15. GC: t_R 6.45 min. IR (Film): 3455*s*, 2979*s*, 2935*s*, 2859*s*, 2665*w*, 2132*w*, 2029*w*, 1887*w*, 1732*s*, 1449*s*, 1373*s*, 1244*s*, 1179*s*, 1085*s*, 1047*s*, 947*s*, 853*m*, 819*m*, 785*m*, 743*m*. ^{1}H-NMR (300 MHz, $CDCl_3$): 4.18 (*q*, ^3J = 7.1, MeCH_2); 2.87 (*s*, OH); 2.24 (*dd*, ^3J = 11.9, ^3J = 3.9, H–C(1)); 2.07–1.99 (*m*, H_{eq}–C(3), H_{eq}–C(6)); 1.81–1.67 (*m*, H_{ax}–C(3), H_{ax}–C(6)); 1.43–1.17 (*m*, CH_2(4), CH_2(5)); 1.27 (*t*, ^3J = 7.1, *Me*CH_2). ^{13}C-NMR (75.4 MHz, $CDCl_3$): 175.3 (C=O); 70.5 (*t*, ^1J(C,D) = 21.7, C(2)); 60.6 (MeCH$_2$); 51.3 (C(1)); 33.6 (C(3)); 28.1 (C(5)); 25.1 (C(6)); 24.4 (C(4)); 14.2 (*Me*CH$_2$). EI-MS: 173 (3, M^+, $[C_9H_{15}DO_3]^+$), 155 (7, $[M-H_2O]^+$), 145 (48, $[M-C_2H_4]^+$), 128 (30), 116 (7), 102 (45), 101 (100), 82 (75), 73 (99).

3. *(±)*-cis- *and (±)*-trans-*2-(Hydroxymethyl)cyclohexan-1-ols* ((±)-**4** and (±)-**5**, resp.). To a soln. of (±)-**1** (8.23 g, 47.5 mmol) in Et_2O (150 ml) $LiAlD_4$ (2.0 g) was added in portions at 0° over 30 min. Then, the mixture was refluxed overnight, after usual workup, continuously extracted with Et_2O, evaporated, and distilled (100°/1 Torr) to yield (±)-**4** (5.42 g, 86%). With an analogous procedure, (±)-**2** (5.29 g, 30.5 mmol) in Et_2O (100 ml) and $LiAlD_4$ (1.28 g, 30.5 mmol) gave (±)-**5** (2.76 g, 68%).

Data of (±)-**4**: White amorphous solid. M.p. 48–50°. R_f (AcOEt) 0.25. GC: t_R 5.9 min. IR (Film): 3360*s* (br.), 2929*s*, 2862*s*, 2669*m*, 2197*m*, 2089*s*, 1929*w*, 1447*s*, 1295*s*, 1251*s*, 1215*s*, 1151*s*, 1091*s*, 942*s*, 821*m*, 778*m*. ^{1}H-NMR (300 MHz, $CDCl_3$): 3.11, 2.95 (2*s*, OH); 1.77 (*m*, H–C(2)); 1.72–1.53 (*m*, 4 H); 1.50–1.21 (*m*, 4 H). ^{13}C-NMR (75.4 MHz, $CDCl_3$): 69.9 (*t*, ^1J(C,D) = 21.3, C(1)); 66.2 (*quint.*, ^1J(C,D) = 21.7, *C*D_2OH); 42.4 (C(2)); 32.9 (C(6)); 24.9 (C(3)); 23.5 (C(4)); 20.4 (C(5)). EI-MS: 133 (< 1, M^+, $[C_7H_{11}D_3O_2]^+$), 115 (22, $[M-H_2O]^+$), 97 (48, $[M-2\ H_2O]^+$), 81 (30), 70 (100), 58 (60).

Data of (±)-**5**: Colorless oil that solidifies in the refrigerator. M.p. 10–15°. R_f (AcOEt) 0.22. GC: t_R 5.9 min. IR (Film): 3323*s* (br.), 2925*s*, 2853*s*, 2665*m*, 2197*m*, 2085*m*, 1650*w*, 1449*s*, 1412*s*, 1332*s*, 1147*s*, 1083*s*, 1002*s*, 961*s*, 906*m*, 855*m*, 817*m*, 484*s*. ^{1}H-NMR (300 MHz, $CDCl_3$): 3.83 (br. *s*, 2 OH); 1.93 (*m*, H–C(2)); 1.76–1.45 (*m*, H_{eq}–C(3), H_{ax}–C(5), H_{eq}–C(5), H_{eq}–C(6)); 1.40–1.12 (*m*, CH_2(4), H_{ax}–C(6)); 0.91 (br. *m*, *dq*-like, H_{ax}–C(3)). ^{13}C-NMR (75.4 MHz, $CDCl_3$): 75.6 (*t*, ^1J(C,D) = 21.3, C(1)); 67.5 (*quint.*, ^1J(C,D) = 21.5, *C*D_2OH); 45.8 (C(2)); 35.2 (C(6)); 27.3 (C(3)); 25.1 (C(4)); 24.5 (C(5)). EI-MS: 133 (< 1, M^+, $[C_7H_{11}D_3O_2]^+$, 115 (22, $[M-H_2O]^+$), 97 (55, $[M-2H_2O]^+$), 81 (26), 70 (100), 58 (62).

4. *(±)*-trans-*3-(2,4-Dinitrophenoxy)-2,4-dioxa-3λ^5-phospha(1,5,5-$^2H_3$)bicyclo[4.4.0]decan-3-ones* ((±)-**6a** and (±)-**6b**). To a soln. of (±)-**4** (533 mg, 4.0 mmol), pyridine (700 μl) and $CHCl_3$ (10 ml), prepared at 0° in a glove-box (N_2 atmosphere), was added dropwise a soln. containing 2,4-dinitrophenyl phosphorodichloridate[8]) (1.51 g, 5.0 mmol) in $CHCl_3$ (8 ml). The mixture was stirred for 12 h at r.t. and then evaporated. Fast CC of the residue (silica gel; abs. hexane/AcOEt 1:3) yielded, from the faster-eluting fraction, the pure axial epimer (±)-**6a** (480 mg, 34%), and, from the slower-eluting one, the equatorial epimer (±)-**6b** (347 mg, 24%).

Data of (±)-**6a**: Opaque crystals. M.p. 125°. R_f (hexane/AcOEt 1:3) 0.54. IR (KBr): 3271*m*, 3123*m*, 3065*w*, 2932*s*, 2864*s*, 2670*w*, 2508*w*, 2264*w*, 1916*w*, 1804*w*, 1613*s*, 1543*s*, 1453*m*, 1346*s*, 1261*s*, 1076*s*, 1022*s*, 934*s*, 899*s*, 835*s*, 782*s*, 661*m*, 607*m*, 485*s*. ^{1}H-NMR (300 MHz, $CDCl_3$): 8.83 (*dd*, ^4J(3′,5′) = 2.7, ^5J(3′,6′) = 1.2, H–C(3′)); 8.46 (*dd*, ^3J(5′,6′) = 9.2, ^4J(5′,3′) = 2.7, H–C(5′)); 8.13 (*dd*, ^3J(6′,5′) = 9.2, ^3J(6′,3′) = 1.2, H–C(6′)); 2.12 (*ddd*, ^2J =

---

[8]) The reagent was prepared according to the procedure described in [7]. It consisted of $Cl_2P(O)OC_6H_3(NO_2)_2$ (*ca.* 60%) (^{31}P-NMR ($CDCl_3$): 5.08 ppm) and $ClP(O)[OC_6H_3(NO_2)_2]_2$ (*ca.* 25%) (^{31}P-NMR ($CDCl_3$): –6.46 ppm).

12.5, ^3J(10eq,9ax) = 3.4, ^3J(10eq,9eq) = 2.0, H_{eq}–C(10)); 2.05 (br. *d*, ^3J(6,7ax) = 11, H–C(6))[9]; 1.94–1.80 (*m*, H_{eq}–C(9)); 1.84–1.70 (*m*, *tt*-like, H_{eq}–C(7), H_{eq}–C(8)); 1.59 (br. *td*, $^2J \approx {}^3J$(10ax,9ax) ≈ 11, ^3J(10ax,9eq) = 3.5, H_{ax}–C(10))[10]; 1.43–1.26 (*m*, *quint.*-like, H_{ax}–C(8), H_{ax}–C(9)); 1.07 (*qd*, $^2J \approx {}^3J$(7ax,6) ≈ ^3J(7ax,8ax) ≈ 12, ^3J(7ax,8eq) = 3.5, H_{ax}–C(7)). ^{13}C-NMR (75.4 MHz, $CDCl_3$): 148.2 (C(1′)); 143.4 (C(4′)); 140.6 (C(2′)); 129.2 (C(5′)); 123.0 (C(6′)); 121.9 (C(3′)); *ca.* 85.0 (br. *m*, C(1))[11]; *ca.* 74.0 (br. *m*, C(5))[11]; 40.7 (*d*, ^3J(6,P) = 6.4, C(6)); 32.3 (*d*, ^3J(10,P) = 8.8, C(10)); 25.2 (C(7)); 24.2 (C(8)); 23.9 (C(9)). ^{31}P-NMR (121.4 MHz, $CDCl_3$, ^{1}H- and ^{2}H-coupled): −14.7 (*s*). ESI-MS MeOH/$CHCl_3$/NaI: 384 (100, $[M+Na]^+$, $[C_{13}H_{12}D_3N_2O_8P+Na]^+$).

Data of (±)-**6b**: Slightly yellowish powder. M.p. 115°. R_f (hexane/AcOEt 1:3) 0.39. IR (KBr): 3450*w*, 3116*m*, 3063*m*, 2939*s*, 2864*m*, 2544*w*, 2258*w*, 2175*w*, 2138*w*, 1936*w*, 1824*w*, 1613*s*, 1546*s*, 1487*s*, 1449*m*, 1417*m*, 1348*s*, 1314*s*, 1171*m*, 1149*m*, 1046*s*, 934*s*, 837*s*, 741*s*, 663*m*, 551*s*, 491*s*. ^{1}H-NMR (300 MHz, $CDCl_3$): 8.80 (*dd*, ^4J(3′,5′) = 2.7, ^5J(3′,6′) = 1.2, H–C(3′)); 8.45 (*dd*, ^3J(5′,6′) = 9.2, ^4J(5′,3′) = 2.7, H–C(5′)); 7.98 (*dd*, ^3J(6′,5′) = 9.2, ^5J(6′,3′) = 1.2, H–C(6′)); 2.33 (br. *d*, ^3J(6,7ax) = 11.5, H–C(6))[9]; 2.20 (*dt*-like, ^2J = 12, ^3J(10eq,9ax) ≈ ^3J(10eq,9eq) ≈ 2, H_{eq}–C(10)); 1.92–1.88 (*m*, H_{eq}–C(9)); 1.84–1.76 (*m*, H_{eq}–C(7), H_{eq}–C(8)); 1.57 (br. *td*, $^2J \approx {}^3J$(10ax,9ay) ≈ 12, ^3J(10ax,9eq) = 3.5, H_{ax}–C(10))[10]; 1.39–1.26 (*m*, *quint.*-like, H_{ax}–C(8), H_{ax}–C(9)); 1.03 (*qd*, $^2J \approx {}^3J$(7ax,6) ≈ ^3J(7ax,8ax) ≈ 12, ^3J(7ax,8eq) = 3.5, H_{ax}–C(7)). ^{13}C-NMR (75.4 MHz, $CDCl_3$): 148.3 (C(1′)); 143.6 (C(4′)); 140.3 (C(2′)); 128.9 (C(5′)); 123.9 (C(6′)); 121.5 (C(3′)); 38.9 (*d*, ^3J(6,P) = 12.4, C(6)); 32.5 (*d*, ^3J(10,P) = 6.3, C(10)); 26.3 (C(7)); 24.1 (C(8)); 23.8 (C(9))[12]. ^{31}P-NMR (121.4 MHz, $CDCl_3$, ^{1}H- and ^{2}H-coupled): −14.1 (*s*). ESI-MS MeOH/$CHCl_3$/NaI: 384 (100, $[M+Na]^+$, $[C_{13}H_{12}D_3N_2O_8P+Na]^+$).

6. N-*[(Benzyloxy)carbonyl]*-O-(trans-*3-oxo-2,4-dioxa-3λ⁵-phospha(1,5,5-²H₃)bicyclo[4.4.0]dec-3-yl)*-L-*serine Methyl Esters* (**8a/8a′** and **8b/8b′**; mixture of diastereoisomers). To a soln. of $POCl_3$ (273 µl, 3.0 mmol) and pyridine (256 µl, 3.17 mmol) in abs. Et_2O (6 ml), prepared at 0° in a glove-box (N_2 atmosphere), a soln. containing (+)-*N*-[(benzyloxy)carbonyl]-L-serine methyl ester (805 mg, 3.17 mmol; *Bachem C-2605*) was added (*in situ* formation of **7**). After stirring for 2 h at 0°, (±)-**4** (400 mg, 3.0 mmol) and pyridine (485 µl, 6.0 mmol) in abs. Et_2O (4 ml) were added, the mixture was stirred for 12 h at r.t. and then evaporated. CC (silica gel; $CHCl_3$/AcOEt 1:1) of the crude product (1.83 g) yielded the faster-eluting axial epimers **8a/8a′** (205 mg, 16%), followed by the equatorial epimers **8b/8b′** (268 mg, 21%), both as mixtures of diastereoisomers (*ca.* 1:1).

Data of **8a/8a′**: Colorless solid. M.p. 125–130°. R_f ($CHCl_3$/AcOEt 1:1) 0.31. ^{1}H-NMR (600 MHz, $CDCl_3$)[13]: 7.38–7.32 (*m*, 5 arom. H); 5.84 (br. *d*, ^3J(NH,2) = 7.7, NH); 5.15, 5.13 (*AB*, ^2J = 12.5, PhCH_2); 4.63 (*X* of *ABX-P*, br. *d*-like, H–C(2)); 4.50 (*A* of *ABX-P*, *tt*-like, H_A–C(3)); 4.38 (*B* of *ABX-P*, *tt*-like, H_B–C(3)); 3.81 (*s*, MeO); 2.04 (*dt*, ^2J = 11.5, ^3J(10′eq,9′ax) ≈ ^3J(10′eq,9′eq) ≈ 2, H_{eq}–C(10)); 1.87 (*m*, br. *t*-like, H–C(6′), H_{eq}–C(9′)); 1.73 (*m*, H_{eq}–C(8′)); 1.63 (*dq*, ^2J = 12, ^3J(7′eq,6′) ≈ ^3J(7′eq,8′ax) ≈ ^3J(7′eq,8′eq) ≈ 2, H_{eq}–C(7′)); 1.50 (*td*, $^2J \approx {}^3J$(10′ax,9′ax) ≈ 12, ^3J(10′ax,9′eq) = 3.5, H_{ax}–C(10′)); 1.31–1-23 (*m*, H_{ax}–C(8′), H_{ax}–C(9′)); 0.87 (br. *qd*, $^2J \approx {}^3J$(7′ax,6′) ≈ ^3J(7′ax,8′ax) ≈ 12, ^3J(7′ax,8′eq) ≈ 4, H_{ax}–C(7′)). ^{13}C-NMR (150.9 MHz, $CDCl_3$): 169.5 (C(1)); 155.7 (OCON); 136.0 (arom. C); 128.5 (2C), 128.2, 128.1 (2 C) (arom. CH); 81.6 (br. *m*, C(1′))[11]; 71.5 (br. *m*, C(5′))[11]; 67.2 (PhCH_2); 66.7, 66.6 (2*d*, ^2J(3,P) = 5.1, 4.9, C(3))[14]; 54.5 (*d*, ^3J(2,P) = 6.7, C(2)); 52.9 (MeO); 40.5 (*d*, ^3J(6′,P) = 5.9, C(6′)); 32.2 (*d*, ^3J(10′,P) = 9.1, C(10′)); 25.2 (C(7′)); 24.2 (C(8′)); 23.8 (C(9′)). ^{31}P{^{1}H}-NMR (121.4 MHz, $CDCl_3$): −7.19 (*m*, $w_{1/2} \approx 16$). ^{31}P{^{1}H}-NMR (242.9 MHz, $CDCl_3$, ^{2}H-coupled): −6.70 (*t*-like, ^3J(P,D) = 3.5). ^{31}P{^{2}H}-NMR (242.9 MHz, $CDCl_3$, ^{1}H-coupled): −6.70 (*t*, ^3J(P,3) = 7.0). ESI-MS MeOH/$CHCl_3$/NaI: 453 (100, $[M+Na]^+$, $[C_{19}H_{23}D_3NO_8P+Na]^+$).

Data of **8b/8b′**: Slightly greenish viscous oil. R_f 0.16 ($CHCl_3$/AcOEt 1:1). ^{1}H-NMR (600 MHz, $CDCl_3$)[13]: 7.36–7.30 (*m*, 5 arom. H); 5.80 (*t*, ^3J(NH,2) ≈ ^4J(NH,3) ≈ 7.1, NH); 5.13 (*s*, PhCH_2); 4.57 (*X* of *ABX-P*, *dd*-like, H–C(2)); 4.50 (*A* of *ABX-P*, *dd*-like, H_A–C(3)); 4.37 (*B* of *ABX-P*, *oct.*-like, H_B–C(3)); 3.77 (*s*, MeO); 2.04 (*m*, br. *dd*-like, H_{eq}–C(10′)); 1.89–1.80 (*m*, H–C(6′), H_{eq}–C(9′)); 1.72 (*m*, br. *d*-like, H_{eq}–C(8′)); 1.63 (*m*, *dq*-like, H_{eq}–C(7′)); 1.43 (*qd*-like, H_{ax}–C(10′)); 1.32–1.20 (*m*, *quint.*-like, H_{ax}–C(8′), H_{ax}–C(9′)); 0.92 (*m*, *qq*-like,

---

[9]) Line broadening due to ^3J(6ax,$^2H_{ax}$–C(5)).

[10]) Line broadening due to ^3J(10ax,$^2H_{ax}$–C(1)).

[11]) Due to ^1J(C,D) and ^2J(C,P), C(1) and C(5) are complex *multiplets* that hardly exceed the noise.

[12]) The signals of C(1) and C(5) could not be detected; see *Footnote 11*.

[13]) In these compounds, diastereoisomerism becomes significant in the ^{1}H-NMR at 600 MHz with respect to 300 MHz. Most of the signals are doubled, but the individual lines are overlapping, an observation that results in an overall lower resolution compared to spectra at 300 MHz. A full interpretation of the data is only made in those cases where the coupling situation was unambiguous.

[14]) Double signals due to diastereoisomerism.

$H_{ax}-C(7')$). ^{13}C-NMR (150.9 MHz, $CDCl_3$): 169.2 (C(1)); 155.8 (OCON); 136.1 (arom. C); 128.5 (2 C), 128.2, 128.1 (2 arom. CH); 81.6 (*t*-like, $^3J(1',P) \approx {}^1J(1',D) \approx 5.5$, C(1')); 71.5 (*quint.*-like, $^3J(5',P) \approx {}^1J(5',D) \approx 5.5$, C(5')); 67.8, 67.9 (*d*, $^2J(3,P) = 5.5$, 6.0, C(3))[14]; 67.1 (PhCH_2); 54.3 (*d*, $^3J(2,P) = 6.6$, C(2)); 52.8 (MeO); 40.5 (*d*, $^3J(6',P) = 6.7$, C(6')); 32.4 (*d*, $^3J(10',P) = 8.2$, C(10')); 25.5 (C(7')); 24.3 (C(8')); 23.8 (C(9')). ^{31}P{^{1}H}-NMR (121.4 MHz, $CDCl_3$): −4.61 (*m*, $w_{1/2} \approx 18$). ^{31}P{^{1}H}-NMR (242.9 MHz, $CDCl_3$, ^{2}H-coupled): −4.10 (*t*-like, $^3J(P,D) = 2.5$). ^{31}P{^{2}H}-NMR (242.9 MHz, $CDCl_3$, ^{1}H-coupled): −4.13, −4.11 (each *t*, $^3J(P,H-C(3)) = 6.5$). ESI-MS MeOH/$CHCl_3$/NaI: 453 (100, $[M+Na]^+$, $[C_{19}H_{23}D_3NO_8P+Na]^+$).

7. *^{31}P-NMR Experiments with δ-Chymotrypsin. Sample Preparation*. The inhibitor (±)-**6a** (0.6 mg, 1.65 μmol) in CD_3CN (60 μl) was quickly added to a soln. of δ-chymotrypsin (40 mg, 1.6 μmol) in D_2O (240 μl)/*Tris* buffer (250 μl, pH 7.8, 0.2M) and mixed on a *Vortex*®. The mixture (yellow due to partial hydrolysis) was transferred into a NMR tube (5 mm i.d.) and kept for 3 d at r.t. in the dark.

^{31}P-NMR Parameters for **9/9'**. *1D-Experiments: Bruker DRX-500*, 202.4 MHz, temp. 330 ± 1K; 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 1.15 s; spectral width 17000 Hz (70 ppm); line-broadening factor 2.0 Hz; 6144 transients. *2D Experiments: Bruker DRX-600*, 242.9 MHz, temp. 330 ± 1K; δ(^{31}P) in ppm rel. to 85% H_3PO_4 (= 0 ppm) as external reference; pulse widths 8.5 μs (90° – ^{1}H), 23.7 μs (90° – ^{31}P); relaxation delay 1.5 s; acquisition time 0.213 s; spectral width 1440 Hz (2.4 ppm, (F_1, ^{1}H)), 1950 Hz (8.0 ppm, (F_2,^{31}P)); window function in F_1 and F_2 = sine 2; time domain in $F_1 = 31$. 1000 transients. 2D H-1/X correlation *via* heteronuclear zero- and double-quantum coherence; phase sensitive using TPPI with decoupling during acquisition; peak-type selection by means of gradient pulses with coherence selection step after t_1.

REFERENCES

[1] W. Ganci, E. J. M. Meier, F. Merckling, G. Przibille, U. Ringeisen, P. Rüedi, *Helv. Chim. Acta* **1997**, *80*, 421.
[2] S. Furegati, W. Ganci, G. Przibille, P. Rüedi, *Helv. Chim. Acta* **1998**, *81*, 1127.
[3] H. C. Froede, I. B. Wilson in 'The Enzymes', Ed. P. D. Boyer, Academic Press New York. 3rd ed. 1971, Vol. 5, p. 87; T. Selwood, S. R. Feaster, M. J. States, A. N. Pryor, D. M. Quinn, *J. Am. Chem. Soc.* **1993**, *115*, 10477.
[4] F. H. Westheimer, *Acc. Chem. Res.* **1968**, *1*, 70; R. F. Hudson, C. Brown, *Acc. Chem. Res.* **1972**, *5*, 204; P. Gillepsie, F. Ramirez, I. Ugi, D. Marquarding, *Angew. Chem., Int. Ed.* **1973**, *12*, 109; S. Tripett, *Pure Appl. Chem.* **1974**, *40*, 595.
[5] N. K. Schaffer, C. S. May, W. H. Summerson, *J. Biol. Chem.* **1953**, *202*, 67; D. M. Blow, J. J. Birktoft, B. S. Hartley, *Nature* **1969**, *221*, 337; D. M. Blow, *Acc. Chem. Res.* **1976**, *9*, 145; L. Polgar, in ' Hydrolytic Enzymes', Eds. A. Neuenberger, K. Brocklehurst, Elsevier, Amsterdam, 1987.
[6] R. Wolfenden, *Acc. Chem. Res.* **1972**, *5*, 10; G. E. Lienhard, *Science* **1973**, *180*, 149.
[7] C. A. Bunton, E. J. Fendler, J. H. Fendler, *J. Am. Chem. Soc.* **1967**, *89*, 1221.

Received August 23, 2000