Structure−Activity Relationship of Diaryl Phosphonate Esters as Potent Irreversible Dipeptidyl Peptidase IV Inhibitors

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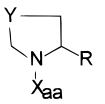
The previously reported diphenyl 1-(*S*)-prolylpyrrolidine-2(*R,S*)-phosphonate (**5**) was used as a lead compound for the development of potent and irreversible inhibitors of dipeptidyl peptidase IV (DPP IV, EC 3.4.14.5). The synthesis of a series of diaryl 1-(*S*)-prolylpyrrolidine-2(*R,S*)-phosphonates with different substituents on the aryl rings (hydroxyl, methoxy, acylamino, sulfonylamino, ureyl, methoxycarbonyl, and alkylaminocarbonyl) started from the corresponding phosphites. A good correlation was found between the electronic properties of the substituent and the inhibitory activity and stability. The most striking divergence of this correlation was the high potency combined with a high stability of the 4-acetylamino-substituted derivative **11e**. This compound shows low cytotoxicity in human peripheral blood mononuclear cells and also has favorable properties in vivo. Therefore bis(4-acetamidophenyl) 1-(*S*)-prolylpyrrolidine-2(*R,S*)-phosphonate (**11e**) is considered as a major improvement and will be a highly valuable DPP IV inhibitor for further studies on the biological function of the enzyme and the therapeutic value of its inhibition.

Introduction

Dipeptidyl peptidase IV (DPP IV, EC 3.4.14.5) is a highly specific exopeptidase with serine-type mechanism protease activity, cleaving off dipeptides from the aminoterminus of peptides with proline at the penultimate position. It is constitutively expressed on epithelial or endothelial cells of a variety of different tissues and is also found in body fluids. In the hematopoietic system DPP IV was identified as the leukocyte antigen CD26,[1] where its expression depends strongly on the differentiation and activation status. T cells with a high surface density of CD26 have been shown to be responsible for proliferation in response to recall antigen in vitro.

Several inhibitors of DPP IV are described belonging to different chemical classes, most of them dipeptide analogues with a proline mimic in P-1 (Chart 1). Pyrrolidides and thiazolidides[2,3] **1** are reversible, competitive inhibitors with inhibition constants in the micromolar range. Pyrrolidine-2-nitriles[4,5] **2** are reversible inhibitors in the submicromolar range. Diacylhydroxylamines[6] **3** are irreversible inhibitors, and boronic acids[7] (e.g., Pro-boroPro **4**) are very potent and specific transition-state analogues with a reversible, slow-binding inhibition. Unfortunately they are unstable in aqueous solution at neutral pH, due to a cyclization reaction between the free amino group of the P-2 amino acid and the boronic acid.[8] The pinanediol esters of these boronic acids are highly potent inhibitors not only because of hydrolysis to the free boronic acid but also because they are active on themselves.[8] Dipeptide diphenyl phosphonates[9,10] (e.g., diphenyl 1-(*S*)-prolylpyrrolidine-2(*R,S*)-phosphonate hydrochloride, (*S*)-Pro-(*S,R*)-Pro^{P}(OPh)$_2$, **5**) are potent, specific, and irreversible inhibitors leading to a phosphonylated serine at the active site of the enzyme. In this case, the recovery of activity of DPP IV was very slow, i.e., only around 10% was regained after 4 weeks.[11] Recently it was shown that peptides containing a N-terminal X-X-Pro sequence also present DPP IV inhibitory activity, like the HIV-1 Tat protein and the shorter Tat(1−9) fragment as well as other synthetic X-X-Pro peptides with a minimum length of 6 amino acids.[12]

Chart 1



	X_{aa}	Y	R
1	Ile	S	H
2	Ile	CH_2	CN
3	Phe	CH2	CO-NH-O-COR
4	Pro	CH_2	$B(OH)_2$
5	Pro	CH_2	$P(O)(OPh)_2$

Several biological functions for DPP IV are reported. The unique proteolytic specificity points to participation in peptide metabolism. Dipeptide cleavage has been proven for several biologically active peptides,[13,14] e.g., the CC-chemokine RANTES.[15] Moreover, DPP IV is essential for intestinal and renal transport of proline-containing peptides.[16,17] The ability to interact with proteins of the extracellular matrix[18] might be important during cell migration. The function of DPP IV in the immune system is thoroughly investigated.[19] Triggering of CD26 provides a costimulatory signal for T cell receptor (TcR/CD3)-mediated T cell activation, leading to IL-2 production, IL-2 receptor upregulation, and T cell proliferation. The use of specific DPP IV

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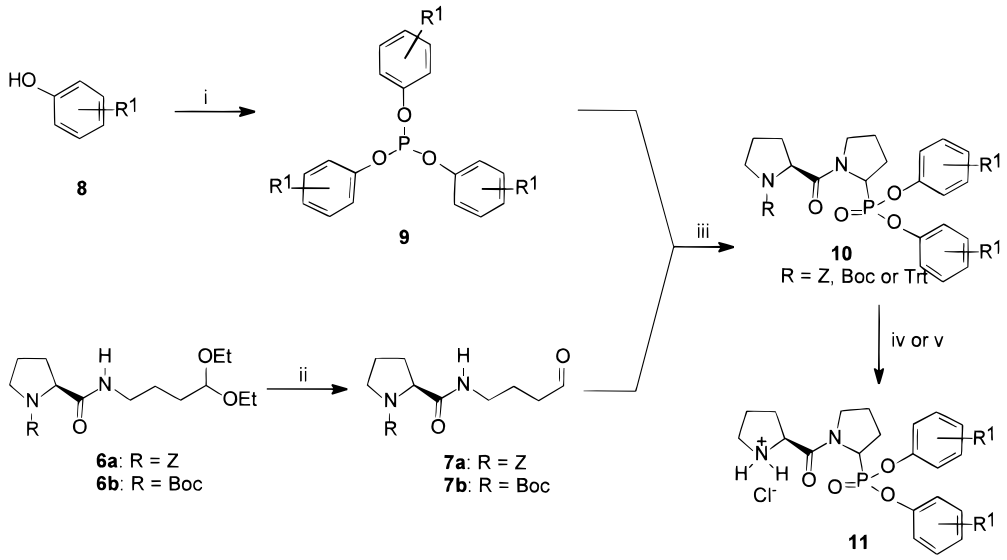
Scheme 1[a]



[a] Reagents: (i) PCl_3; (ii) HCl; (iii) HOAc, 90 °C, 2 h; (iv) HCl/EtOAc (1 M); (v) H_2, Pd/C. For **8**−**11**: R^1 = (**a**) H, (**b**) 4-OMe, (**c**) 4-OAc (4-OH for **11**), (**d**) 3-NHAc, (**e**) 4-NHAc, (**f**) 4-$NHSO_2Me$, (**g**) 3-$NHCONH_2$, (**h**) 4-(*N*-Bz-Gly-NH), (**i**) 4-(*N*-Z-Gly-NH) [4-(H-Gly-NH) for **11**], (**j**) 4-(*N*-Z-(*S*)-Ala-NH) [4-(H-(*S*)-Ala-NH) for **11**], (**k**) 4-((*S*)-Pyr-NH), (**l**) 4-[(2*S*)-$MeO_2CCH(NHAc)CH_2$], (**m**) 4-COOMe, (**n**) 4-($CONHCH_2COOEt$), (**o**) 4-[$CONH(CH_2)_2COOMe$], (**p**) 4-($CONH(CH_2)_2CH_3$).

inhibitors (Pro-boroPro **4**) suppresses T cell proliferation in vitro[7] and decreases antibody production in mice immunized with BSA.[20] Several DPP IV inhibitors also suppressed alkyldiamine-induced arthritis in a dose-dependent manner.[21] The disadvantage of the boronic acid **4** is that frequent administration was required to maintain serum DPP IV activity at a low level, due to rapid inactivation of the inhibitor. In this respect, the dipeptide diphenyl phosphonate **5** is advantageous because of profound and long-lasting irreversible inhibition and higher stability ($t_{1/2}$ in human citrated plasma = 5 h).[11] Intravenous injection of a single dose of **5** (1, 5, or 10 mg) in rabbits causes a decrease in plasma DPP IV activity with more than 80%, and it takes more than 20 days for complete recovery. Not only plasma DPP IV was inhibited, but also DPP IV in circulating lymphocytes and peripheral tissues.[22] This prompted us to investigate the role of DPP IV/CD26 in alloantigen-driven T cell activation. Heart transplantation in rats gives an early (24-h) increase in cellular CD26 expression followed by a rise in DPP IV serum activity, which peaked at day 6, i.e., before the time of actual graft loss. Diphenyl phosphonate **5** abrogates acute rejection and prolongs cardiac allograft survival from 7 to 14 days. This was associated with severely impaired host cytotoxic T lymphocyte responses in vitro.[23]

Pyrrolidine-2-nitrile inhibitors of DPP IV have shown to inhibit HIV-1 infection in a T cell line, suggesting that DPP IV/CD26 enzymatic acitivity may play a role in facilitating HIV-1 infection of human $CD4^+$ T cells at the entry process.[24]

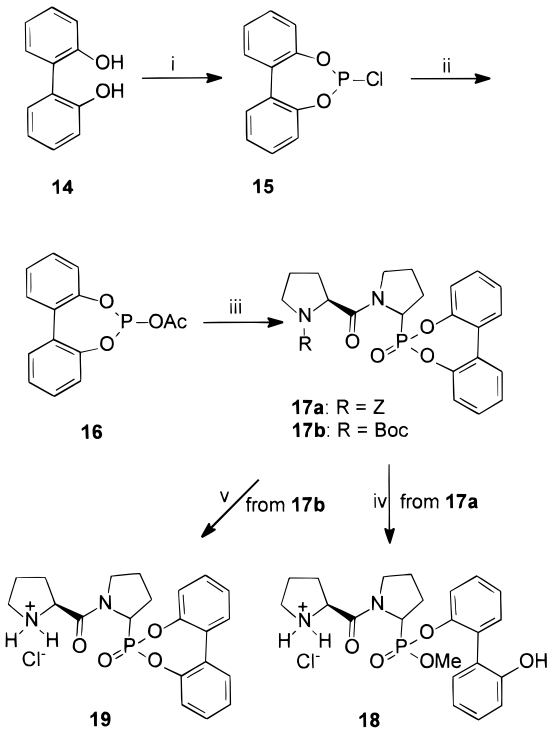
The recent results showing that DPP IV/CD26 plays an important role in the mechanism of allograft rejection in vivo prompted us to investigate in more detail the structure−activity relationship of dipeptide diphenyl phosphonates as inhibitors. We have shown previously[11] in a study on the role of the P-2 amino acid that proline at this position gave one of the most active, and certainly the most stable, inhibitors in human citrated plasma. It was described[9] that bis(4-chlorophenyl) phosphonates are more active than their nonsubstituted congeners. These data prompted us to investigate the influence of different functional groups on the inhibitory activity of these phosphonates. We synthesized prolylpyrrolidine diphenyl phosphonates, substituted on the phenyls with hydroxyl, methoxy, acylamino, sulfonylamino, ureyl, methoxycarbonyl, and alkylaminocarbonyl groups. The phenyl esters were also replaced by other groups with good leaving group capacities such as trichloroethyl and trifluoroethyl. The DPP IV inhibitory activity in vitro and in vivo, the stability, and the specificity of these compounds will be described.

Results and Discussion

Synthesis. The synthesis of a series of prolylpyrrolidine diaryl phosphonates started with the coupling of 4-aminobutyraldehyde diethyl acetal to N-protected proline, activated as mixed anhydride with isobutyl chloroformate, as published previously.[10] The acetal **6** was hydrolyzed with HCl, and the crude aldehyde **7** was treated with the corresponding triaryl phosphite **9** in acetic acid to give diastereoisomeric mixtures of the protected prolylpyrrolidine diaryl phosphonate **10**[10] (Scheme 1). Deprotection using standard methods afforded the final compounds **11**.

For the preparation of substituted phenyl phosphonates, it was necessary to prepare some commercially unavailable phenols. The 4-(methylsulfonylamino)-phenol (**8f**) was prepared from the corresponding sulfonyl chloride and 4-aminophenol. Likewise, the 4-(acylamino)phenols **8h**−**k** were prepared by condensation of the corresponding carboxylic acid with 4-aminophenol using the mixed anhydride method. *N*-Acetyl-L-tyrosine methyl ester (**8l**) was prepared as described.[25] The glycine derivative **8n** was obtained after condensation of glycine ethyl ester with 4-hydroxybenzoic acid using diphenyl phosphorylazide (DPPA). The synthesis of the 4-hydroxybenzoic acid amides **8o** and **8p** was accomplished by coupling of 4-acetoxybenzoic acid with the corresponding amine using the mixed anhydride method

Scheme 2[a]



[a] Reagents: (i) PCl_3; (ii) AcOH, Et_3N; (iii) **7**, HOAc, 90 °C, 2 h; (iv) H_2, Pd/C; (v) HCl/EtOAc (1 M).

followed by mild alkaline hydrolysis of the phenyl esters **12** and **13**.[26] The triaryl phosphites **9** were then synthesized from the corresponding substituted phenols **8** and phosphorus trichloride.

Cyclic N-protected 2,2′-biphenylyl derivatives **17a** and **17b** were prepared by reacting 2,2′-biphenylylacetyl phosphite (**16**) with aldehyde **7a** or **7b** in acetic acid (Scheme 2). Attempted removal of benzyloxycarbonyl (Z) protection from phosphonate **17a** by hydrogenolysis with a Pd/C catalyst in methanol resulted in the opening of the dioxaphosphepan ring to give the mixed methyl aryl ester **18**. Deprotection of the Boc derivative **17b** with HCl/EtOAc yielded free 2,2′-biphenylyl phosphonate **19**.

Phosphonate analogues with 2,2,2-trichloroethyl and 2,2,2-trifluoroethyl as leaving groups[27] were prepared similarly to the aryl esters by reaction of aldehyde **7a** with the corresponding phosphites, followed by deprotection of the resulting phosphonates **20** and **21** to the target compounds **22** and **23**.

Generally, with this method we obtained a mixture of diastereoisomers of the phosphonates **10**. To obtain the pure diastereoisomers of **5**, it was necessary to introduce a trityl protection after removal of the Z-protection, which resulted in easily separable isomers (**10a(*S*,*R*)** and **10a(*S*,*S*)**). We suppose that the most active isomer has the *R* configuration at the carbon atom next to phosphorus, and this was confirmed by comparison of the relative mobility on TLC, optical activity, ^{1}H NMR spectrum, and biological activity with that of (*S*)-Ile-(*R*)-Pro^P(OPh)$_2$. The configuration of this reference compound was unambiguously determined with X-ray crystallography.[10]

DPP IV Inhibition and Stability of the Inhibitors. In a previous study[11] on the role of the P-2 amino acid in dipeptide diphenyl phosphonates, we showed that proline in this position gives one of the most potent inhibitors. A major advantage of **5** was its greater stability in human citrated plasma compared to the other dipeptide derivatives. The stability of **5** in plasma equals the stability in buffer, whereas for the other compounds the stability in plasma was reduced compared to buffer. This reflects the relative stability of a Pro-Pro amino acid sequence to proteolytic breakdown and indicates that the decrease in activity of **5** is mainly caused by hydrolysis of the phosphonate ester. To increase the inhibitory activity while retaining the stability, we introduced several substituents on the phenyl rings that act as leaving groups. Powers et al. showed that a 4-chlorophenoxy group improved the inhibition considerably in the case of DPP IV[9], but not for other serine proteases.[28] The influence of electron-donating or -withdrawing substituents on enzyme inhibition and stability was investigated as shown in Table 1. All compounds were irreversible inhibitors of DPP IV, probably due to the formation of a phosphonylated serine at the active site of the enzyme. The inactivation rate constants were calculated from experimental IC_{50} values and were in reasonable agreement with the measured inactivation rate constants, where available.

We observed a good correlation between the electron-withdrawing properties of the substituent and its activity (Figure 1). Introduction of an electron-donating substituent (4-OH, **11c**) decreases potency, whereas an electron-withdrawing substituent (4-methoxycarbonyl, **11m**) increases potency. The most striking divergence in the correlation between the Hammett constant and the inhibitory activity is the strong inhibition caused by the 4-(acetylamino)phenyl (**11e**) and the 4-(methylsulfonylamino)phenyl (**11f**) phosphonate esters. These compounds have similar Hammett constants compared to the diastereoisomeric mixture of **5** but are nevertheless about 100 times more potent. This indicates that other features might also be important for binding. The equal potency of the 3-acetylamino (**11d**) and the 4-acetylamino (**11e**) also points to an extra interaction of **11e** with the active site of the enzyme, considering the fact that **11d** should be much more active because of a higher electron-withdrawing potential. The possibility of an extra interaction was further investigated by introduction of other acylamino substituents. The 4-glycylamino (**11i**) and the 4-alanylamino (**11j**) have a comparable activity but are considerably less stable in plasma than the 4-acetylamino (**11e**)-substituted diphenyl phosphonate. We suppose that this is due to proteolytic cleavage of **11i** and **11j** to the 4-amino derivative, which will be considerably less active because of its strong electron-donating properties (Hammett constant of -0.57 gives a calculated IC_{50} of 975 μM). This conversion of a very active to an almost inactive molecule gives a rapid decrease of the apparent IC_{50} value, reflected in a short half-life. To prove this assumption, we measured the stability of **11i** and **11j** in buffer (50 mM Tris·HCl, pH = 8). The half-lives of **11i** (96 ± 7 min) and **11j** (99 ± 4 min) in buffer are higher than in plasma and equal the half-life of the *N*-benzoyl-protected 4-glycylamino derivative **11h** in plasma. This indicates that the breakdown of **11i** and

Table 1. Potency and Stability of DPP IV Inhibitors[a]

compd	R_1	config at Pro^P C(2)	IC_{50} (μM)	k_{calc} (M^{-1} s^{-1})	$t_{1/2}$ (min) in plasma
5	H	*R,S*	32 ($n = 1$)	2.4×10^1	250 ± 10
11a	H	*R*	15 ± 3 ($n = 3$)	5.1×10^1	300 ± 30
11a	H	*S*	$>10^4$		
11b	4-MeO	*R,S*	22 ± 11 ($n = 4$)	3.5×10^1	470 ± 90
11c	4-HO	*R,S*	190 ± 80 ($n = 3$)	4.1	$\gg 200$
11d	3-AcNH	*R,S*	0.8 ± 0.1 ($n = 2$)	9.6×10^2	220 ± 30
11e	4-AcNH	*R,S*	0.4 ± 0.2 ($n = 5$)	1.9×10^3	320 ± 140
11f	4-$MeSO_2NH$	*R,S*	0.40 ± 0.02 ($n = 2$)	1.9×10^3	150 ± 30
11g	3-H_2NCONH	*R,S*	2.3 ± 0.3 ($n = 2$)	3.3×10^2	210 ± 80
11h	4-(*N*-Bz-Gly-NH)	*R,S*	0.7 ± 0.3 ($n = 2$)	1.1×10^3	93 ± 3
11i	4-(H-Gly-NH)	*R,S*	0.5 ± 0.1 ($n = 2$)	1.5×10^3	28 ± 3
11j	4-(H-(*S*)-Ala-NH)	*R,S*	0.6 ± 0.2 ($n = 2$)	1.3×10^3	8 ± 1
11k	4-((*S*)-Pyr-NH)	*R,S*	5.0 ± 0.8 ($n = 2$)	1.5×10^2	170 ± 30
11l	4-[(2*S*)-$MeO_2CCH(NHAc)CH_2$]	*R,S*	1.4 ± 0.5 ($n = 4$)	5.5×10^2	190 ± 150
11m	4-MeO_2C	*R,S*	0.016 ± 0.004 ($n = 2$)	4.8×10^4	19 ± 1
11n	4-(EtO_2CCH_2NHCO)	*R,S*	0.023 ± 0.007 ($n = 2$)	3.3×10^4	35 ± 2
11o	4-[$MeO_2C(CH_2)_2NHCO$]	*R,S*	0.036 ± 0.006 ($n = 4$)	2.1×10^4	26 ± 1
11p	4-[$CH_3(CH_2)_2NHCO$]	*R,S*	0.03 ± 0.01 ($n = 2$)	2.6×10^4	12 ± 1
18	P(OMe)(OC_6H_4(2-OH-C_6H_4))	*R,S*	47 ± 18 ($n = 2$)	1.6×10^1	140 ± 80
19	P-2,2′-biphenylyl	*R,S*	31 ± 6 ($n = 2$)	2.5×10^1	6 ± 1
22	P(OCH_2CCl_3)$_2$	*R,S*	1800 ($n = 1$)	4.3×10^{-1}	
23	P(OCH_2CF_3)$_2$	*R,S*	9100 ($n = 1$)	8.5×10^{-2}	

[a] IC_{50} values were determined after 15-min preincubation with DPP IV at 37 °C. The listed values are the average of *n* independent measurements ± the standard deviation. Functional stability in plasma was estimated by fitting the inverse of the apparent IC_{50} values versus time with a single-exponential decay. The half-life is listed ± the standard error of fit.

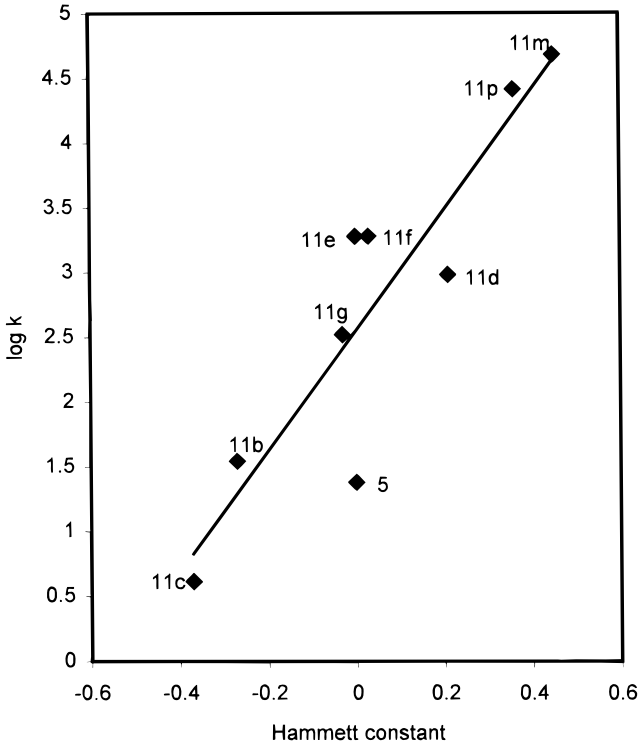


Figure 1. Correlation between Hammett constant and log k_{calc}. A good correlation (log $k = 4.6504\sigma + 2.5472$, $r^2 = 0.82$) was observed between the Hammett constant and log k_{calc}, the most striking divergence being the difference between the unsubstituted **5** and the 4-acetylamino **11e**.

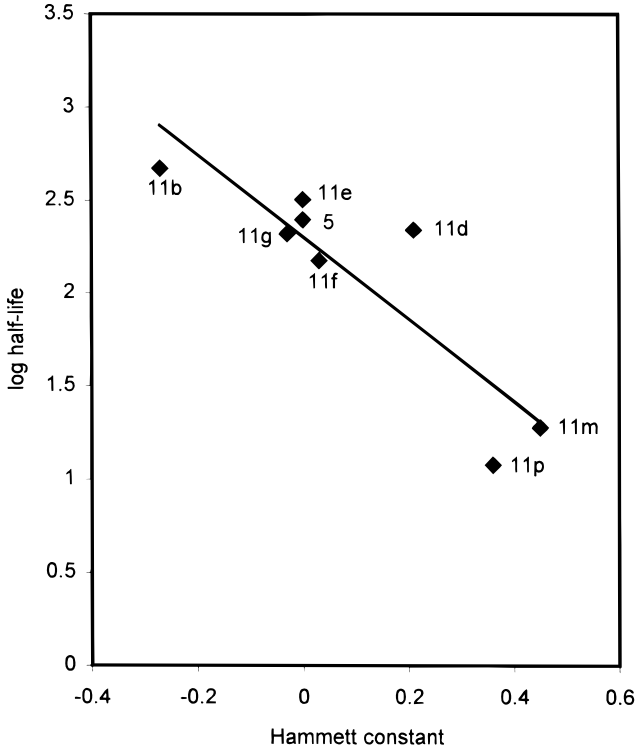


Figure 2. Correlation between Hammett constant and log half-life. A good correlation (log $t_{1/2} = -2.2138\sigma + 2.3043$, $r^2 = 0.77$) was observed between the Hammett constant and log $t_{1/2}$.

11j in plasma is caused by hydrolysis of the phosphonate ester and also by cleavage of the amino acid substituent and that this cleavage can be overcome with a *N*-benzoyl protection.

The half-life of the inhibitors seems to correlate also with the Hammett constant (Figure 2). Compounds with similar electronic properties (e.g., **5** and **11e**) have a similar stability in plasma. This is in agreement with the fact that the decrease in activity is mainly caused by hydrolysis of the phosphonate esters. The more electron-withdrawing derivatives (methoxycarbonyl and alkylaminocarbonyl, **11m**−**p**) are very potent inhibitors (IC_{50} = 16 nM) but are also unstable ($t_{1/2}$ = 19 min). The only exceptions to the correlation between electronic properties and half-life are the aminoacyl derivatives **11i** and **11j** (vide supra) and the dioxaphosphepan ring **19**. The chemical instability of this ring is reflected in its short half-life in plasma. This compound has the same potency as **5**, but its hydrolyzed derivative shows

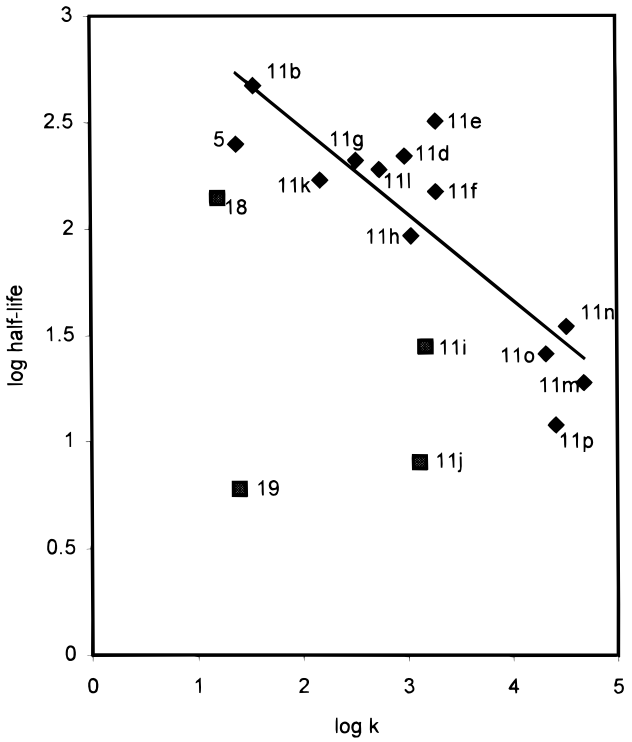


Figure 3. Correlation between log k_{calc} and log half-life. A good correlation (log $t_{1/2} = -0.4051(\log k) + 3.2899$, $r^2 = 0.75$) was observed between log half-life and log k. Compounds **11i**, **11j**, **18**, and **19** were left out. Compounds **11i** and **11j** have a considerably shorter half-life than could be expected, presumably because they suffer from another metabolism, extra to the phosphonate ester hydrolysis (see Discussion). Compounds **18** and **19** cannot be compared directly to the other diaryl phosphonate esters.

no inhibitory activity. On the contrary, the *O*-methyl derivative **18** is almost as potent and stable as **5**. The phosphonate esters of trichloroethanol (**22**) and trifluoroethanol (**23**) have rather disappointing inhibitory activities.

Unfortunately, the correlation between electronic properties and inhibitory activity and between electronic properties and stability results also in an inverse correlation between inhibitory activity and stability (Figure 3). This means that more active compounds are also more unstable. Therefore, the higher potency (IC_{50} = 0.4 μM) and equal stability ($t_{1/2}$ = 320 min) of the paracetamol-substituted phenyl phosphonate **11e** is a major improvement to the unsubstituted **5**.

In Vitro Cytotoxicity and Efficacy in Human Peripheral Blood Mononuclear Cells (PBMC). On the basis of inhibition potency, stability in plasma, and synthesis efficiency, compounds **11e** and **11n** were selected for further in vitro and in vivo studies. Both compounds were evaluated in human peripheral blood mononuclear cells (PBMC) and did not show cytotoxicity in freshly isolated mononuclear cells or phytohemagglutinin-stimulated blasts when concentrations up to 100 μM were used. Under these circumstances, more than 90% of the DPP IV activity in cell lysates as well as in supernatants was inhibited. This is in contrast with the results obtained for the active diastereoisomer of the unsubstituted diphenyl phosphonate **11a**, where no satisfactory inhibition of DPP IV activity could be reached without cytotoxic effects on PBMC cultures. The compounds **11e** and **11n** are therefore promising tools for further studies on a cellular level. The irreversible mechanism of inhibition overcomes the rather limited stability of the compounds in biological media.

DPP IV Inhibition in Vivo. We reported previously[22] that a single intravenous injection of **5** (0.3−5 mg/kg) in rabbits caused a decrease in plasma DPP IV activity with more than 80%, and it took more than 20 days for complete recovery.

In rats, a comparable dose per weight intravenously did not result in a sufficient inhibition of circulating DPP IV, because adequate inhibition could not be reached without severe systemic toxicity. However, a combination of subcutaneous and intraperitoneal injections of **11a** allowed us to bring plasma DPP IV to less than 15% of pretreatment values. The observation that monotherapy with the diastereoisomeric mixture of **11a** (**5**) not only significantly prolonged graft acceptance upon alloantigen challenge[23] but occasionally also caused systemic toxicity and more often local ulcerations stimulated the in vivo testing of **11e** and **11n** in rabbits, rats, and mice.

In rabbits, the higher in vitro potency of **11n** compared to **11e** was also observed. The IC_{50} values for inhibition of plasma DPP IV in rabbits upon single intravenous injection was below 20 μg/kg for **11n** and was around 0.2 mg/kg for **11e**. In this species single intravenous injection of 2 mg/kg of **11n** inhibited plasma DPP IV activity for more than 95% during at least 24 h, without side effects.

In rats, **11e** as well as **11n** could keep plasma DPP IV activity below 10% of pretreatment values by daily subcutaneous injection of 50 mg/kg (initial dose 100 mg/kg), without any sign of acute systemic or local toxicity.

In mice, a pharmacologically useful inhibition was only obtained with **11n** and not with **11a** and **11e**. The molecular basis for the large interspecies differences in efficiency remains to be elucidated. Due to the higher intrinsic stability, **11e** was selected for further investigations in rat disease models.

Specificity for DPP IV. The specificity for DPP IV/CD26 was examined by comparison with inhibition rates for other proteases (Table 2). Prolyl endopeptidase (PEP), dipeptidyl peptidase II (DPP II), membrane alanyl aminopeptidase (MAAP) and elastase were used for compounds **11a**, **11e**, and **11n**, also studied in vitro and in vivo. At concentrations equal to the IC_{50} value for DPP IV, these compounds were specific, because only a small inhibition was noticed for **11n** with PEP (15%) and DPP II (22%). This is remarkably specific knowing that DPP II is quite similar to DPP IV, being also a serine-type exopeptidase, cleaving off dipeptides from the amino terminus, with some specificity for proline at the penultimate position. At a higher concentration (1 mM, 40000-fold higher than the IC_{50} value for DPP IV) **11n** gives complete inhibition of PEP and elastase, some inhibition of DPP II, and almost no inhibition of MAAP. Some other compounds were only used to measure their inhibitory potency for PEP. Here also, a concentration equal to the IC_{50} value for DPP IV gives a very small inhibition of PEP. In general, 50% inhibition of PEP was obtained at concentrations 1250−1700-fold higher and complete inhibition at concentrations

Table 2. Specificity of the Inhibitors **11** for DPP IV/CD26[a]

		PEP		DPP II		MAAP		elastase	
compd	R_1	IC_{50}	1 mM	IC_{50}	1 mM	IC_{50}	1 mM	IC_{50}	1 mM
11a	H (*R* at carbon next to phosphorus)	107	71	102	73	100	83	100	82
11d	3-AcNH	89	50						
11e	4-AcNH	96	60	100	83	99	96	106	105
11f	4-$MeSO_2NH$	73	4						
11h	4-(N-Bz-Gly-NH)	92	32						
11j	4-(H-(S)-Ala-NH)	88	47						
11l	4-[(2*S*)-$MeO_2CCH(NHAc)CH_2$]	102	35						
11m	4-MeO_2C	73	4						
11n	4-(EtO_2CCH_2NHCO)	85	1	78	66	104	94	102	6
11o	4-[$MeO_2C(CH_2)_2NHCO$]	83	0						
11p	4-[$CH_3(CH_2)_2NHCO$]	97	5						

[a] The effect of the different inhibitors **11** on the activity of prolyl endopeptidase (PEP), dipeptidyl peptidase II (DPP II), membrane alanyl aminopeptidase (MAAP), and elastase was evaluated at the IC_{50} concentration for DPP IV (see Table 1) and at 1 mM. The residual enzyme activity is given as a percentage toward a control experiment without inhibitor.

2500−62500-fold higher than the IC_{50} value for DPP IV. So, we can say that the selectivity index is at least 1250 compared to PEP and much higher for the other enzymes.

Conclusion

The recent result that a dipeptide diphenyl phosphonate inhibitor of DPP IV/CD26 abrogated acute rejection and prolonged cardiac allograft survival in rats prompted us to investigate in more detail the structure−activity relationship of these compounds using diphenyl 1-(*S*)-prolylpyrrolidine-2(*R*,*S*)-phosphonate as a lead compound. We synthesized a series of diaryl 1-(*S*)-prolylpyrrolidine-2(*R*,*S*)-phosphonates with different substituents on the aryl rings (hydroxyl, methoxy, acylamino, sulfonylamino, ureyl, methoxycarbonyl, and alkylaminocarbonyl) and measured their DPP IV inhibitory potency and stability in plasma. All compounds were irreversible inhibitors. A good correlation was observed between the electron-withdrawing properties of the substituents and the inhibition of DPP IV. The methoxycarbonyl- and alkylaminocarbonyl-substituted derivatives were the most potent inhibitors with IC_{50} values around 20 nM and inactivation rate constants around 3000 M^{-1} s^{-1}. The same correlation was also observed between the electron-donating properties of the substituents and the stability in plasma. The most potent inhibitors are also the most unstable compounds. A notable exception is the good stability of the 4-(acetylamino)phenyl phosphonate ester **11e** ($t_{1/2}$ = 320 min), together with a higher potency than could be expected (IC_{50} = 0.4 μM, k = 1900 M^{-1} s^{-1}). Therefore, this compound together with the very potent **11n** were further investigated in vitro and in vivo. These inhibitors showed no cytotoxicity in human peripheral blood mononuclear cells in concentrations up to 100 μM. The IC_{50} values of **11e** and **11n** for inhibition of plasma DPP IV in rabbits upon single intravenous injection were around 0.2 mg/kg and below 20 μg/kg, respectively. The compounds also showed no acute systemic or local toxicity, as was observed with the unsubstituted lead compound **5**.

Due to the higher stability of **11e** compared to **11n**, we believe that bis(4-acetamidophenyl) 1-(*S*)-prolylpyrrolidine-2(*R*,*S*)-phosphonate (**11e**) is considered as a major improvement and will be a highly valuable inhibitor for further studies on the biological function of the enzyme and the therapeutic value of its inhibition. The advantage of this compound compared to the pyrrolidine-2-nitrile reversible inhibitors is its long-lasting irreversible inhibition. Moreover, it is more stable than the frequently used boronic acid inhibitors.

Experimental Section

General. 4-Methoxyphenol (**8b**), 1-(3-hydroxyphenyl)urea (**8g**), methyl 4-hydroxybenzoate (**8m**), triphenyl phosphite (**9a**), 2,2′-biphenol (**14**), L-proline, L-tyrosine, 4-aminophenol, glycine, L-pyroglutamic acid, 4-hydroxybenzoic acid, glycine ethyl ester hydrochloride, β-alanine, and all common chemicals were purchased from Acros Chimica N.V., Belgium. 3-Acetamidophenol (**8d**), 4-aminobutyraldehyde diethyl acetal, PCl_3, diphenyl phosphorylazide (DPPA), and 4-acetoxybenzoic acid were obtained from Sigma-Aldrich Chemie BV, Belgium. 4-Acetamidophenol (paracetamol) (**8e**) was obtained from Sterling Organics Ltd., England. Purity of all new synthesized compounds was checked by TLC, ^{1}H NMR, ^{13}C NMR, or MS. The final products were checked by TLC, ^{1}H NMR, ^{13}C NMR, FAB-MS, and(or) elemental analysis. Thin-layer chromatography was performed with POLYGRAM SIL G/UV_{254} plates precoated with silica gel (Machinery-Nagel GmbH, Germany) using EtOAc−petroleum ether, EtOAc−MeOH, or *n*-BuOH−AcOH−H_2O (4:1:1) mixtures as eluent. Silica gel H, 5−40 μm (Fluka, Switzerland), was used for preparative vacuum column chromatography. The NMR spectra were recorded on a Varian EM360L or a Brucker Avance DRX 400 spectrometer. Mass spectra were recorded on a VG 70-SEQ spectrometer. Optical rotation was measured on a Perkin-Elmer 241 polarimeter. Melting points were determined on a digital melting points apparatus (Electrothermal) and are uncorrected. Elemental analyses were performed at The Institute of Organic Chemistry, University of Tübingen, Germany.

Z- and Boc-protected amino acids were prepared according to standard procedures[29] using Z-Cl and $(Boc)_2O$, respectively. β-Alanine methyl ester was synthesized by treatment of the amino acid in methanolic HCl similarly to described procedure.[29] The preparation of 4-(Z-(*S*)-prolyl)aminobutyraldehyde diethyl acetal (**6a**) was described earlier.[10] Tris(2,2,2-trichloroethyl) phosphite and tris(2,2,2-trifluoroethyl) phosphite were prepared as described.[30] 2-Chlorodibenzo[*d*,*f*]-1,3,2-dioxaphosphepan (**15**) was prepared as described.[31]

4-(Boc-*(S)*-prolyl)aminobutyraldehyde Diethyl Acetal (6b). To a solution of Boc-*(S)*-proline (10 mmol, 2.14 g) in dry $CHCl_3$ (20 mL) was added Et_3N (10 mmol, 1.4 mL) with stirring at −10 °C followed by isobutyl chloroformate (10 mmol, 1.31 mL). After 20 min, 4-aminobutyraldehyde diethyl acetal (10 mmol, 1.73 mL) was added, and the mixture was stirred overnight (−10 °C to room temperature). Chloroform was removed, the residue was distributed between EtOAc (100 mL) and water (100 mL), and the organic layer was dried ($MgSO_4$) and purified by column chromatography: yield 2.96 g (83%), oil; ^{1}H NMR ($CDCl_3$) δ 1.0−2.4 (m, 23H, 3- and 4-CH_2 (Pro),

CH_2CH_2, $C(CH_3)_3$, CH_3), 2.9−3.9 (m, 9H, 5-CH_2, NCH_2, OCH_2 and $(EtO)_2CH$), 4.25 (m, 1H, 2-CH), 4.5 (t, 1H, NH).

4-(Methylsulfonylamino)phenol (8f). To a suspension of 4-aminophenol (200 mmol, 21.8 g) in MeOH (250 mL) was added CH_3SO_2Cl (100 mmol, 7.75 mL) at 10−15 °C with stirring. The resulting solution was stirred for 1 h at room temperature and evaporated. The residue was suspended in 1 N HCl (250 mL); the solid was filtered, washed with water, and dried in a vacuum over NaOH: yield 13.6 g (81%); mp 165−166 °C; ^{1}H NMR (DMSO) δ 2.85 (s, 3H, CH_3S), 6.70 (d, $2H_{arom}$), 7.10 (d, $2H_{arom}$), 8.70 (s, 1H, OH), 8.85 (s, 1H, NH).

Synthesis of 4-(Acylamino)phenols 8h−k. General Procedure. To a solution of carboxylic acid (100 mmol) in DMF (100 mL), was added Et_3N (100 mmol, 14 mL) at −10 °C, followed by isobutyl chloroformate (100 mmol, 13 mL). After 0.5 h at this temperature, 4-aminophenol (115 mmol, 12.5 g) was added and the mixture was stirred overnight (−10 °C to room temperature). After dilution with 1 N HCl (500 mL), the precipitate was collected by filtration and washed on the filter with water, $NaHCO_3$ solution, water, and, finally, ether. The product was dried and, if necessary, recrystallized from an appropriate solvent.

***N*²-Benzoyl-*N*-(4-hydroxyphenyl)glycinamide (8h):** recrystallized from EtOH−acetone; yield 35%; mp 244−245 °C; ^{1}H NMR (DMSO) δ 4.05 (d, 2H, CH_2CO), 6.5−8.0 (m, $9H_{arom}$), 8.7 (m, 1H, NH), 9.15 (s, 1H, OH), 9.75 (s, 1H, NH).

***N*²-(Benzyloxycarbonyl)-*N*-(4-hydroxyphenyl)glycinamide (8i):** yield 44%; mp 184−185 °C; ^{1}H NMR (DMSO) δ 3.80 (d, 2H, CH_2CO), 6.5−7.5 (m, 10H, $9H_{arom}$ and NH), 8.85 (s, 1H, OH), 9.40 (s, 1H, NH).

***N*²-(Benzyloxycarbonyl)-*N*-(4-hydroxyphenyl)-*(S)*-alaninamide (8j):** yield 73%; mp 97−100 °C; ^{1}H NMR (DMSO) δ 1.36 (d, 3H, CH_3), 4.29 (m, 1H, CH of L-Ala), 5.00 (s, 2H, CH_2), 6.00 (m, 1H, NH), 6.35−7.38 (m, 9H, H_{arom}), 8.69 (br s, 1H, OH), 9.24 (br s, 1H, NHCO)

***N*-(4-Hydroxyphenyl)-*(S)*-pyroglutamamide (8k):** yield 64%; mp 303−305 °C dec; ^{1}H NMR (DMSO) δ 1.7−2.7 (m, 4H, CH_2CH_2), 4.2 (m, 1H, CHCO), 6.65 (d, $2H_{arom}$), 7.40 (d, $2H_{arom}$), 7.8 (s, 1H, NH), 9.1 (br s, 1H, OH), 9.7 (s, 1H, NH).

***N*-(4-Hydroxybenzoyl)glycine Ethyl Ester (8n).** To a solution of 4-hydroxybenzoic acid (100 mmol, 13.8 g) in DMF (70 mL) was added H-Gly-OEt·HCl (100 mmol, 14 g) followed by DPPA (100 mmol, 21.6 mL) and Et_3N (200 mmol, 28 mL) with stirring at 0 °C. The mixture was stirred overnight (0 °C to room temperature), Et_3N·HCl was removed by filtration, and the filtrate was evaporated to one-half of the initial volume. The residue was mixed with 5% $NaHCO_3$ (200 mL) and extracted with EtOAc (500 mL). The organic layer was washed with brine, dried ($MgSO_4$), and evaporated until crystallization. The mixture was diluted with ether (100 mL), and the crystals were collected: yield 9 g (40%); mp 205−209 °C; ^{1}H NMR (DMSO) δ 1.25 (t, 3H, CH_3), 4.05 (m, 4H, CO_2CH_2 and $COCH_2N$), 6.8 (d, $2H_{arom}$), 7.75 (d, $2H_{arom}$), 8.35 (tr, 1H, NH), 9.7 (s, 1H, OH).

Synthesis of 4-Hydroxybenzoic Acid Amides 8o and 8p. General Procedure. To a solution of 4-acetoxybenzoic acid (100 mmol, 18 g) in THF (250 mL) was added Et_3N (100 mmol, 14 mL) followed by isobutyl chloroformate (100 mmol, 13 mL) with stirring at −10 °C. After 15 min the corresponding amine (free base or HCl salt) was added followed by Et_3N (100 mmol, 14 mL) in case of a hydrochloride. The mixture was stirred overnight (−10 °C to room temperature), the solid was removed by filtration, the filtrate was evaporated, and the residue was crystallized from an ether−hexane mixture. 4-Acetoxybenzoic acid amide **12** or **13** thus obtained (70 mmol) was dissolved in MeOH (240 mL); H_2O (90 mL); was added followed by saturated $NaHCO_3$ solution (120 mL). An excess of $NaHCO_3$ precipitated. The heterogeneous mixture was stirred for 4 h at room temperature, methanol was removed in a vacuum, the residue was extracted with EtOAc (250 mL), and the organic layer was washed with 1 N HCl and brine, then dried ($MgSO_4$), and evaporated. The residue crystallized from ether or was purified on a silica gel column.

***N*-(4-Acetoxybenzoyl)-*β*-alanine methyl ester (12):** yield 69%; mp 84−88 °C; ^{1}H NMR ($CDCl_3$) δ 2.35 (s, 3H, $COCH_3$), 2.6 (t, 2H, CO_2CH_2), 3.7 (m, 5H, NCH_2 and CO_2CH_3), 7.15 (m, 3H, $2H_{arom}$ and NH), 7.75 (d, $2H_{arom}$).

***N*-*n*-Propyl-4-acetoxybenzoylamide (13):** yield 73%; mp 94−98 °C; ^{1}H NMR ($CDCl_3$) δ 0.9 (t, 3H, CH_3), 1.6 (m, 2H, CH_2), 2.25 (s, 3H, $COCH_3$), 3.4 (m, 2H, NCH_2), 7.1 (m, 3H, $2H_{arom}$ and NH), 7.8 (d, $2H_{arom}$).

***N*-(4-Hydroxybenzoyl)-*β*-alanine methyl ester (8o):** yield 65%; mp 124−126 °C; ^{1}H NMR (DMSO) δ 2.55 (t, 2H, CO_2CH_2), 3.7 (m, 5H, NCH_2 and CO_2CH_3), 6.9 (m, 3H, $2H_{arom}$ and NH), 7.55 (d, $2H_{arom}$), 8.7 (br s, 1H, OH).

***N*-*n*-Propyl-4-hydroxybenzoylamide (8p):** yield 78%; oil; ^{1}H NMR (DMSO) δ 1.1 (t, 3H, CH_3), 1.55 (m, 2H, CH_2), 3.3 (m, 2H, NCH_2), 6.9 (m, 3H, $2H_{arom}$ and NH), 7.65 (d, $2H_{arom}$), 8.5 (br s, 1H, OH); MS (FAB^+) *m*/*z* 180 $(M + H)^+$.

Synthesis of Triaryl Phosphites 9. General Procedure. To a stirred solution of the corresponding phenol (90 mmol) and Et_3N (90 mmol, 12.6 mL) in dry DMF (30 mL) was added a solution of PCl_3 (30 mmol, 2.62 mL) in dry $CHCl_3$ (10 mL) dropwise at 0 °C. The mixture was stirred overnight (0 °C to room temperature) and diluted with $CHCl_3$ (500 mL), and the resulting solution was washed with water (2 × 500 mL). The organic layer was dried ($MgSO_4$) and evaporated and the residue, eventually together with the product insoluble in both layers, was purified by column chromatography with an EtOAc−methanol or an EtOAc−petroleum ether mixture as eluents.

Tris(4-methoxyphenyl) phosphite (9b): yield 85%, oil; ^{1}H NMR ($CDCl_3$) δ 3.51 (s, 9H, OCH_3), 6.51−7.15 (m, $12H_{arom}$).

Tris(4-acetoxyphenyl) phosphite (9c): yield 55%, oil; ^{1}H NMR ($CDCl_3$) δ 2.21(s, 9H, CH_3), 7.05 (m, 12H, H_{arom}).

Tris(3-acetamidophenyl) phosphite (9d): yield 42%, solid foam; ^{1}H NMR (DMSO) δ 2.1 (s, 9H, $COCH_3$), 6.7−7.7 (m, $12H_{arom}$), 9.7 (s, 3H, NH).

Tris(4-acetamidophenyl) phosphite (9e): yield 37%, solid foam; ^{1}H NMR (DMSO) δ 2.1 (s, 9H, $COCH_3$), 6.9−7.7 (dd, $12H_{arom}$), 9.2 (s, 3H, NH).

Tris[4-(methylsulfonylamino)phenyl] phosphite (9f): yield 60%, solid foam; ^{1}H NMR (DMSO) δ 2.95 (s, 9H, SO_2CH_3), 7.2 (m, $12H_{arom}$), 9.15 (s, 3H, NH).

Tris(3-ureylphenyl) phosphite (9g): yield 30%, solid foam; ^{1}H NMR (DMSO) δ 5.85 (m, 6H, $CONH_2$), 6.5−7.5 (m, $12H_{arom}$), 8.65 (s, 3H, NH).

Tris[4-(*N*-benzoylglycylamino)phenyl] phosphite (9h): yield 90%; mp 210 °C dec; ^{1}H NMR (DMSO) δ 4.1 (d, 6H, $COCH_2N$), 6.9−8.0 (m, $27H_{arom}$), 8.5 (m, 3H, NH), 9.9 (s, 3H, NH).

Tris[4-(*N*-benzyloxycarbonylglycylamino)phenyl] phosphite (9i): yield 76%; mp 173 °C dec; ^{1}H NMR (DMSO) δ 3.85 (d, 6H, $COCH_2N$), 5.05 (s, 6H, CH_2Ph), 6.5−8.0 (m, 30H, NH and H_{arom}), 9.8 (br s, 3H, NH).

Tris[4-(*N*-benzyloxycarbonyl-*(S)*-alanylamino)phenyl] phosphite (9j): yield 85%, solid foam; ^{1}H NMR (DMSO) δ 1.42 (d, 9H, CH_3), 4.29 (m, 3H, CH), 5.01(s, 6H, CH_2Ph), 6.45−7.15 (d, 3H, NH), 7.25 (m, 27H, H_{arom}), 9.76 (br s, 3H, NH).

Tris[4-((*S*)-pyroglutamylamino)phenyl] phosphite (9k): yield 31%, solid foam; ^{1}H NMR (DMSO) δ 1.8−2.8 (m, 12H, CH_2CH_2), 4.25 (m, 3H, COCHN), 7.1 (d, $6H_{arom}$), 7.65 (d, $6H_{arom}$), 8.25 (s, 3H, NH), 10.3 (s, 3H, NH).

Tris[4-(*S*)-(2-methoxycarbonyl-2-acetamidoethyl)phenyl] phosphite (9l): yield 73%, solid foam; ^{1}H NMR (DMSO) δ 2.0 (s, 9H, $COCH_3$), 3.1 (d, 6H, $ArCH_2$), 3.7 (s, 9H, CO_2CH_3), 4.8 (m, 3H, COCHN), 6.6 (d, 3H, NH), 7.1 (br s, $12H_{arom}$).

Tris[4-(methoxycarbonyl)phenyl] phosphite (9m): yield 57%, oil; ^{1}H NMR ($CDCl_3$) δ 3.95 (s, 9H, CO_2CH_3), 6.65−8.05 (m, $12H_{arom}$).

Tris{4-[(ethoxycarbonyl)methylaminocarbonyl]-phenyl} phosphite (9n): yield 70%, solid foam; ^{1}H NMR ($CDCl_3$) δ 1.3 (t, 9H, CH_3), 3.9−4.3 (m, 12H, $COCH_2N$ and OCH_2), 6.7−8.0 (m, 15H, NH and H_{arom}).

Tris{4-[2-(methoxycarbonyl)ethylaminocarbonyl]-phenyl} phosphite (9o): yield 71%, solid foam; ^{1}H NMR

($CDCl_3$) δ 2.65 (t, 6H, CO_2CH_2), 3.7 (m, 15H, NCH_2 and CO_2CH_3), 7.1 (d, $6H_{arom}$), 7.9 (m, 9H, H_{arom} and NH).

Tris[4-(*n*-propylaminocarbonyl)phenyl] phosphite (9p): yield 54%; mp 207 °C; ^{1}H NMR (DMSO) δ 1.1 (t, 9H, CH_3), 1.6 (m, 6H, CH_2), 3.25 (m, 6H, CH_2N), 7.15 (d, $6H_{arom}$), 8.1 (m, 9H, NH and H_{arom}).

Separation of the Diastereoisomers of 10a: Diphenyl 1-(Trityl-*(S)*-prolyl)pyrrolidine-2*(R)*-phosphonate (10a(*S,R*)) and Diphenyl 1-(Trityl-*(S)*-prolyl)pyrrolidine-2*(S)*-phosphonate (10a(*S,S*)). A solution of diphenyl 1-(Z-*(S)*-prolyl)-pyrrolidine-2*(R,S)*-phosphonate[10] (13.1 mmol, 7 g) in MeOH (130 mL) was hydrogenated over 10% Pd/C for 6 h at room temperature. The catalyst was removed on Celite, the filtrate was evaporated, and the residue was distributed between EtOAc (100 mL) and saturated $NaHCO_3$ solution (50 mL). The organic layer was dried ($MgSO_4$) and evaporated. The resulting oil (2 g) was dissolved in dry $CHCl_3$ (50 mL); Et_3N (5 mmol, 0.7 mL) was added with stirring at room temperature followed by trityl chloride (5 mmol, 1.39 g). The solution was stirred overnight and washed with water (100 mL); the organic layer was dried, evaporated, and purified by column chromatography (petroleum ether/EtOAc, 4/1 to 2/1). The faster running isomer was **10a(*S,R*)**.

Isomer 10a(*S,R*): yield 0.78 g (9%), solid foam; ^{1}H NMR ($CDCl_3$) δ 0.7−2.8 (m, 10H, 3-CH_2 and 4-CH_2, 5-CH_2 Pro), 3.3−3.5 (m, 2H, 5-CH_2 Pro^P), 4.0 (dd, 1H, J = 8.95, 2.8 Hz, 2-CH Pro), 5.15 (m, 1H, 2-CH Pro^P), 6.9−7.6 (m $25H_{arom}$); $[\alpha]_D^{20}$ = −79.5° (*c* 1, $CHCl_3$); MS (FAB$^+$) *m/z* 643 (M + H)$^+$.

Isomer 10a(*S,S*): yield 0.82 g (11%), solid foam; ^{1}H NMR ($CDCl_3$) δ 0.7−2.6 (m, 8H, 3-CH_2 and 4-CH_2), 2.7−3.7 (m, 4H, 5-CH_2), 3.9 (m, 0.5H, 2-CH Pro^P), 4.2 (dd, 1H, J = 13.5, 7.4 Hz, 2-CH Pro), 5.05 (br t, 0.5H, 2-CH Pro^P), 6.9−7.8 (m, $25H_{arom}$); $[\alpha]_D^{20}$ = −27.2° (*c* 1, $CHCl_3$); MS (FAB$^+$) *m/z* 643 (M + H)$^+$.

Synthesis of *N*-Boc- or *N*-*Z*-Protected Diaryl 1-((*S*)-Prolyl)pyrrolidine-2*(R,S)*-phosphonates 10. General Procedure. Diethyl acetal **6a** or **6b** (10 mmol) was dissolved in a mixture of THF (60 mL) and 0.5 N HCl (30 mL, 15 mmol) with stirring, at room temperature. After 2 h ether (90 mL) was added with stirring, and the mixture was neutralized with an excess of solid $NaHCO_3$ (ca. 5 g). The organic layer was separated, dried ($MgSO_4$), and evaporated. The residue (crude aldehyde **7a** or **7b**) was dissolved in acetic acid (30 mL) together with the corresponding triaryl phosphite **9** (10 mmol), and the resulting solution was stirred at 85−90 °C for 1.5−2 h. After cooling to room temperature, acetic acid was evaporated; the residue was dissolved in chloroform (250 mL) and washed with water (200 mL), saturated $NaHCO_3$ solution (100 mL), and water (100 mL). The organic layer was dried ($MgSO_4$) and evaporated, and the residue was purified by column chromatography with an ethyl acetate−petroleum ether or ethyl acetate−methanol mixture as eluents.

Bis(4-methoxyphenyl) 1-(*tert*-butyloxycarbonyl-*(S)*-prolyl)pyrrolidine-2*(R,S)*-phosphonate (10b): yield 33%, oil; ^{1}H NMR ($CDCl_3$) δ 1.15−2.65 (m, 17H, $C(CH_3)_3$, 3-CH_2 and 4-CH_2), 3.67 (s, 6H, OCH_3), 3.15−4.3 (m, 4H, 5-CH_2), 4.35−5.4 (m, 2H, 2-CH), 6.45−7.35 (m, $8H_{arom}$).

Bis(4-acetoxyphenyl) 1-(benzyloxycarbonyl-*(S)*-prolyl)pyrrolidine-2*(R,S)*-phosphonate (10c): yield 50%, solid foam; ^{1}H NMR ($CDCl_3$) δ 1.65−2.85 (m, 8H, 3-CH_2 and 4-CH_2), 2.29 (s, 6H, $COCH_3$), 3.00−3.85 (m, 4H, 5-CH_2), 4.1−4.85 (m, 2H, 2-CH), 5.15 (s, 2H, CH_2), 6.92 (m, $8H_{arom}$), 7.33 (s, 5H, H_{arom}).

Bis(3-acetamidophenyl) 1-(benzyloxycarbonyl-*(S)*-prolyl)pyrrolidine-2*(R,S)*-phosphonate (10d): yield 31%, solid foam; ^{1}H NMR ($CDCl_3$) δ 1.0−2.7 (m, 8H, 3-CH_2 and 4-CH_2), 2.1 (s, 6H, CH_3CO), 4.0−3.2 (m, 4H, 5-CH_2), 4.3−5.1 (m, 2H, 2-CH), 5.1 (br s, 2H, CH_2Ph), 6.6−7.7 (m, $13H_{arom}$), 9.1 (s, 2H, NH); MS (FAB$^+$) *m/z* 649 (M + H)$^+$.

Bis(4-acetamidophenyl) 1-(benzyloxycarbonyl-*(S)*-prolyl)pyrrolidine-2*(R,S)*-phosphonate (10e): yield 60%, solid foam; ^{1}H NMR ($CDCl_3$) δ 1.6−2.7 (m, 8H, 3-CH_2 and 4-CH_2), 2.1 (s, 6H, CH_3CO), 3.3−3.8 (m, 4H, 5-CH_2), 4.3−5.1 (m, 2H, 2-CH), 5.1 (br s, 2H, CH_2Ph), 6.8−7.5 (m, $13H_{arom}$), 8.8 (s, 2H, NH); MS (FAB$^+$) *m/z* 649 (M + H)$^+$.

Bis[4-(methylsulfonylamino)phenyl] 1-(benzyloxycarbonyl-*(S)*-prolyl)pyrrolidine-2*(R,S)*-phosphonate (10f): yield 66%, solid foam; ^{1}H NMR ($CDCl_3$) δ 1.2−2.7 (m, 8H, 3-CH_2 and 4-CH_2), 2.8 (s, 6H, CH_3SO_2), 3.3−3.8 (m, 4H, 5-CH_2), 4.3−5.2 (m, 4H, 2-CH and CH_2Ph), 6.8−7.4 (m, $13H_{arom}$), 8.0 (s, 2H, NH); MS (FAB$^+$) *m/z* 721 (M + H)$^+$.

Bis(3-ureylphenyl) 1-(benzyloxycarbonyl-*(S)*-prolyl)-pyrrolidine-2*(R,S)*-phosphonate (10g): yield 8%, solid foam; ^{1}H NMR ($CDCl_3$) δ 1.3−2.4 (m, 8H, 3-CH_2 and 4-CH_2), 2.8−3.8 (m, 4H, 5-CH_2), 4.3−5.2 (m, 4H, 2-CH and CH_2Ph), 5.6 (m, 4H, NH_2), 6.8−7.5 (m, $13H_{arom}$), 8.65 (br s, 2H, NH); MS (FAB$^+$) *m/z* 651 (M + H)$^+$.

Bis[4-(*N*-benzoylglycylamino)phenyl]-1-(benzyloxycarbonyl-*(S)*-prolyl)pyrrolidine-2*(R,S)*-phosphonate (10h): yield 47%, solid foam; ^{1}H NMR ($CDCl_3$) δ 1.2−2.7 (m, 8H, 3-CH_2 and 4-CH_2), 3.2−3.8 (m, 4H, 5-CH_2), 4.0 (m, 4H, NCH_2-CO), 4.4−5.0 (m, 2H, 2-CH), 5.1 (br s, 2H, CH_2Ph), 6.6−8.3 (m, 25H, $23H_{arom}$ and NH), 9.5 (br s, 2H, NH); MS (FAB$^+$) *m/z* 887 (M + H)$^+$.

Bis[4-(*N*-benzyloxycarbonylglycylamino)phenyl]-1-(benzyloxycarbonyl-*(S)*-prolyl)pyrrolidine-2*(R,S)*-phosphonate (10i): yield 19%, solid foam; ^{1}H NMR ($CDCl_3$) δ 1.2−2.6 (m, 8H, 3-CH_2 and 4-CH_2), 3.2−4.1 (m, 8H, 5-CH_2 and $COCH_2N$), 4.3−5.2 (m, 8H, 2-CH and CH_2Ph), 6.1 (m, 2H, NH), 6.7−7.6 (m, $23H_{arom}$), 8.95 (br s, 2H, NH); MS (FAB$^+$) *m/z* 947 (M + H)$^+$.

Bis[4-(*N*-benzyloxycarbonyl-*(S)*-alanylamino)phenyl]-1-(benzyloxycarbonyl-*(S)*-prolyl)pyrrolidine-2*(R,S)*-phosphonate (10j): yield 40%; mp 200−203 °C; ^{1}H NMR ($CDCl_3$) δ 1.35 (d, 6H, CH_3), 1.65−2.35 (m, 8H, 3-CH_2 and 4-CH_2), 2.95−3.95 (m, 4H, 5-CH_2), 4.0−4.65 (m, 4H, 2-CH, CH), 5.00 (s, 6H, CH_2Ph), 6.20 (br, 2H, NH), 7.04 (s, 23H, H_{arom}), 9.25 (br, 2H, NH); MS (FAB$^+$) *m/z* 975 (M + H)$^+$.

Bis[4-((*S*)-pyroglutamylamino)phenyl]-1-(benzyloxycarbonyl-*(S)*-prolyl)pyrrolidine-2*(R,S)*-phosphonate (10k): yield 25%; mp 170 °C dec; ^{1}H NMR ($CDCl_3$) δ 1.2−2.9 (m, 16H, 3-CH_2 and 4-CH_2), 3.55 (m, 4H, 5-CH_2), 4.1 (m, 2H, 5-CH Pyr), 4.3−5.2 (m, 4H, 2-CH and CH_2Ph), 6.7−8.1 (m, 15H, NH and $13H_{arom}$), 9.55 (br s, 2H, NH); MS (FAB$^+$) *m/z* 787 (M + H)$^+$.

Bis[4-*(S)*-(2-methoxycarbonyl-2-acetamidoethyl)phenyl] 1-(benzyloxycarbonyl-*(S)*-prolyl)pyrrolidine-2*(R,S)*-phosphonate (10l): yield 57%, solid foam; ^{1}H NMR ($CDCl_3$) δ 1.3−2.7 (m, 8H, 3-CH_2 and 4-CH_2), 1.9 (s, 6H, $COCH_3$), 3.1 (d, 4H, CH_2Ar), 3.7 (s, 6H, $COOCH_3$), 3.2−3.9 (m, 4H, 5-CH_2), 4.5−5.0 (m, 4H, 2-CH), 5.1 (br s, 2H, CH_2Ph), 6.1 (d, 2H, NH), 7.0 (s, $8H_{arom}$), 7.3 (br s, 5H, C_6H_5); MS (FAB$^+$) *m/z* 821 (M + H)$^+$.

Bis[4-(methoxycarbonyl)phenyl] 1-(*tert*-butyloxycarbonyl-*(S)*-prolyl)pyrrolidine-2*(R,S)*-phosphonate (10m): yield 5.5%, solid foam; ^{1}H NMR ($CDCl_3$) δ 1.05−2.55 (m, 8H, 3-CH_2 and 4-CH_2), 1.42 (s, 9H, $C(CH_3)_3$), 3.33−4.06 (m, 4H, 5-CH_2), 3.83 (s, 6H, OCH_3), 4.45 (m, 1H, 2-CH Pro), 5.04 (m, 1H, 2-CH Pro^P), 7.25 (m, 4H, H_{arom}), 7.96 (m, 4H, H_{arom}).

Bis{4-[(ethoxycarbonyl)methylaminocarbonyl]phenyl} 1-(benzyloxycarbonyl-*(S)*-prolyl)pyrrolidine-2*(R,S)*-phosphonate (10n): yield 32%, solid foam; ^{1}H NMR ($CDCl_3$) δ 1.2−2.7 (m, 8H, 3-CH_2 and 4-CH_2), 1.3 (t, 6H, CH_3), 3.2−3.9 (m, 4H, 5-CH_2), 4.0 (m, 4H, NCH_2CO), 4.2 (q, 4H, OCH_2), 4.3−5.1 (m, 2H, 2-CH), 5.1 (br s, 2H, CH_2Ph), 6.9−7.8 (m, 15H, $13H_{arom}$ and NH); MS (FAB$^+$) *m/z* 793 (M + H)$^+$.

Bis{4-[2-(methoxycarbonyl)ethylaminocarbonyl]-phenyl} 1-(benzyloxycarbonyl-*(S)*-prolyl)pyrrolidine-2*(R,S)*-phosphonate (10o): yield 22%, solid foam; ^{1}H NMR ($CDCl_3$) δ 1.2−2.8 (m, 8H, 3-CH_2 and 4-CH_2), 2.65 (t, 4H, CH_2CO_2), 3.2−3.8 (m, 8H, 5-CH_2 and CH_2N), 3.75 (s, 6H, CO_2CH_3), 4.3−5.2 (m, 4H, 2-CH and CH_2Ph), 6.7−7.9 (m, 15H, $13H_{arom}$ and NH); MS (FAB$^+$) *m/z* 793 (M + H)$^+$.

Bis[4-(*n*-propylaminocarbonyl)phenyl] 1-(benzyloxycarbonyl-*(S)*-prolyl)pyrrolidine-2*(R,S)*-phosphonate (10p): yield 4.3%, solid foam; ^{1}H NMR ($CDCl_3$) δ 0.85 (t, 6H, CH_3), 1.3−2.8 (m, 12H, 3-CH_2, 4-CH_2 and CH_2), 3.1−3.9 (m, 8H, 5-CH_2 and CH_2N), 4.5 (m, 1H, 2-CH Pro), 4.7−5.2 (m, 3H,

CH_2Ph and 2-CH Pro^P), 6.9−8.1 (m, 15H, 13H_{arom} and NH); MS (FAB^+) *m/z* 705 $(M + H)^+$.

Diaryl 1-((S)-Prolyl)pyrrolidine-2-phosphonate Hydrochlorides 11. Procedure A. The diaryl 1-(*tert*-butyloxycarbonyl-*(S)*-prolyl)pyrrolidine-2-phosphonate **10** or diaryl 1-(trityl-*(S)*-prolyl)pyrrolidine-2-phosphonate **10** (5 mmol) was dissolved in a 1 M HCl solution in EtOAc (25 mL), and the solution was stirred for 2 h at room temperature. Dry ether (30 mL) was added, and the mixture was left in the fridge overnight. If the product crystallized, the crystals were collected by filtration; otherwise the oil was triturated in dry ether. The material obtained was dried in a vacuum over NaOH pellets.

Procedure B. The diaryl 1-((*S*)-benzyloxycarbonylprolyl)-pyrrolidine-2-phosphonate **10** (2 mmol) was hydrogenated over Pd/C in methanol (50 mL) for 5−6 h. The catalyst was removed by filtration through Celite, the filtrate was acidified with 1 M HCl/EtOAc (2.2 mL) and evaporated, and the residue was precipitated with dry ether from methanol (5 mL). The resulting product was dried in a vacuum over NaOH pellets.

Diphenyl 1-((*S*)-prolyl)pyrrolidine-2*(R)*-phosphonate hydrochloride (11a(*S,R*)): A; yield 81%; mp 175−177 °C; ^1H NMR ($CDCl_3$) δ 1.6−2.8 (m, 8H, 3-CH_2 and 4-CH_2), 3.3−3.9 (m, 4H, 5-CH_2), 4.8 (m, 1H, 2-CH), 5.0 (m, 1H, 2-CH), 7.2 (m, 5H_{arom}), 7.3 (m, 5H_{arom}), 8.1 (br s, 1H, N^+H), 10.2 (br s, 1H, N^+H); ^{13}C NMR ($CDCl_3$) δ 24.1(4-CH_2), 24.6 (4-CH_2), 26.1 (3-CH_2), 28.8 (3-CH_2), 46.2 (5-CH_2), 47.1 (5-CH_2), 54.5 (d, 2-CH−P, ^1J (C−P) = 162 Hz), 59.1 (2-CH), 120.3, 125.2, 129.7, 150.3 (C_{arom}), 168.2 (CO); $[\alpha]_D^{20}$ = −100.4° (*c* 1, $CHCl_3$). Anal. ($C_{21}H_{25}N_2O_4P$·1.25HCl) C, H, N.

Diphenyl 1-((*S*)-prolyl)pyrrolidine-2*(S)*-phosphonate hydrochloride (11a(*S,S*)): A; yield 88%, solid foam; ^1H NMR ($CDCl_3$) δ 1.7−2.6 (m, 8H, 3-CH_2 and 4-CH_2), 3.2−3.9 (m, 4H, 5-CH_2), 4.75 (m, 1H, 2-CH), 4.95 (m, 0.7H, 2-CH), 5.4 (m, 0.3H, 2-CH), 6.9−7.4 (m, 10H_{arom}), 8.6 (br s, 1H, N^+H), 10.3 (br s, 0.3H, N^+H), 10.6 (br s, 0.7H, N^+H); ^{13}C NMR ($CDCl_3$) δ 22.1, 24.0 (4-CH_2), 23.5, 23.9 (4-CH_2), 26.4, 27.7 (3-CH_2), 28.5, 29.1 (3-CH_2), 45.6, 46.2 (5-CH_2), 46.6, 47.1 (5-CH_2), 54.4, 55.3 (d, 2-CH−P, ^1J (C−P) = 160 Hz), 58.3, 58.7 (2-CH), 120.1, 125.0, 129.4, 149.5 (C_{arom}), 167.3, 168.4 (CO); $[\alpha]_D^{20}$ = 25.9° (*c* 1, $CHCl_3$); MS (FAB^+) *m/z* 401 $(M + H)^+$.

Bis(4-methoxyphenyl) 1-((*S*)-prolyl)pyrrolidine-2*(R,S)*-phosphonate hydrochloride (11b): A; yield 70%; ^1H NMR ($CDCl_3$) δ 1.75−2.60 (m, 8H, 3-CH_2 and 4-CH_2), 3.28−3.65 (m, 4H, 5-CH_2), 3.75 (s, 6H, OCH_3), 5.35−5.50 (m, 2H, 2-CH), 6.70−7.60 (m, 8H, H_{arom}), 8.45 (br s, 1H, N^+H), 10.55 (br s, 1H, N^+H); MS (FAB^+) *m/z* 461 $(M + H)^+$; ^{31}P NMR ($CDCl_3$) δ 16.22, 16.72. Anal. ($C_{23}H_{29}N_2O_6P$·2HCl·$2H_2O$) C, H, N.

Bis(4-hydroxylphenyl) 1-((*S*)-prolyl)pyrrolidine-2*(R,S)*-phosphonate hydrochloride (11c): B; yield 80%; ^1H NMR ($CDCl_3$) δ 1.70−2.68 (m, 8H, 3-CH_2 and 4-CH_2), 3.27−3.75 (m, 4H, 5-CH_2), 4.45−4.95 (m, 2H, 2-CH), 6.55−7.16 (m, 8H, H_{arom}), 7.84 (br s, 2H, OH), 8.65 (br s, 1H, N^+H), 10.18 (br s, 1H, N^+H); MS (FAB^+) *m/z* 433 $(M + H)^+$; ^{31}P NMR (DMSO) δ 17.31, 17.40. Anal. ($C_{21}H_{25}N_2O_6P$·HCl·$1.5H_2O$) C, H, N.

Bis(3-acetamidophenyl) 1-((*S*)-prolyl)pyrrolidine-2*(R,S)*-phosphonate hydrochloride (11d): B; yield 83%, solid foam; ^1H NMR (DMSO) δ 1.6−2.6 (m, 8H, 3-CH_2 and 4-CH_2), 2.1 (s, 6H, $COCH_3$), 3.3−3.8 (m, 4H, 5-CH_2), 4.7 (m, 1H, 2-CH), 4.9 (m, 1H, 2-CH), 6.7−7.6 (m, 8H_{arom}), 8.5 (m, 1H, N^+H), 9.7 (m, 1H, N^+H), 9.9 (m, 2H, CONHPh). Anal. ($C_{25}H_{31}N_4O_6P$·1.5HCl·$0.5H_2O$) C, H, N.

Bis(4-acetamidophenyl) 1-((*S*)-prolyl)pyrrolidine-2*(R,S)*-phosphonate hydrochloride (11e): B; yield 90%, solid foam; ^1H NMR (DMSO) δ 1.6−2.6 (m, 8H, 3-CH_2 and 4-CH_2), 2.1 (s, 6H, $COCH_3$), 3.3−3.8 (m, 4H, 5-CH_2), 4.6 (m, 1H, 2-CH), 4.9 (m, 1H, 2-CH), 7.1 (m, 4H_{arom}), 7.6 (m, 4H_{arom}), 8.7 (m, 1H, N^+H), 10.0 (m, 2H, CONHPh), 10.2 (m, 1H, N^+H); ^{31}P NMR (DMSO) δ 17.48, 17.62. Anal. ($C_{25}H_{31}N_4O_6P$·HCl·$2H_2O$) C, H, N.

Bis[4-(methylsulfonylamino)phenyl] 1-((*S*)-prolyl)pyrrolidine-2*(R,S)*-phosphonate hydrochloride (11f): B; yield 80%, solid foam; ^1H NMR (DMSO) δ 1.55−2.5 (m, 8H, 3-CH_2 and 4-CH_2), 2.95 (s, 6H, SO_2CH_3), 3.3−3.8 (m, 4H, 5-CH_2), 4.55 (m, 1H, 2-CH), 4.85 (m, 1H, 2-CH), 7.15 (m, 8H_{arom}), 8.6 (br s, 1H, N^+H), 9.79 (s, 2H, SO_2NHPh), 10.3 (br s, 1H, N^+H). Anal. ($C_{23}H_{31}N_4O_8PS_2$·HCl) C, H, N.

Bis(3-ureylphenyl) 1-((*S*)-prolyl)pyrrolidine-2*(R,S)*-phosphonate hydrochloride (11g): B; yield 90%, solid foam; ^1H NMR (CD_3OD) δ 1.4−2.5 (m, 8H, 3-CH_2 and 4-CH_2), 3.3−3.8 (m, 4H, 5-CH_2), 4.5 (m, 1H, 2-CH), 4.8 (m, 1H, 2-CH), 6.4−7.2 (m, 6H_{arom}), 7.4 (m, 2H_{arom}); MS (FAB^+) *m/z* 517 $(M + H)^+$. Anal. ($C_{23}H_{29}N_6O_6P$·HCl·$2.3H_2O$) C, H, N.

Bis[4-(*N*-benzoylglycylamino)phenyl] 1-((*S*)-prolyl)-pyrrolidine-2*(R,S)*-phosphonate hydrochloride (11h): B; yield 87%, solid foam; ^1H NMR (DMSO) δ 1.6−2.6 (m, 8H, 3-CH_2 and 4-CH_2), 3.2−3.6 (m, 4H, 5-CH_2), 4.2 (d, 4H, NCH_2-CO), 4.5−4.9 (m, 2H, 2-CH), 6.9−7.9 (m, 20H, 18H_{arom} and NH), 8.5 (m, 1H, N^+H), 10.0 (s, 2H, NH), 10.2 (m, 1H, N^+H); MS (FAB^+) *m/z* 753 $(M + H)^+$. Anal. ($C_{39}H_{41}N_6O_8P$·HCl·$2H_2O$) C, H, N.

Bis[4-(*N*-benzyloxycarbonylglycylamino)phenyl] 1-((*S*)-prolyl)pyrrolidine-2*(R,S)*-phosphonate trihydrochloride (11i): B; yield 81%, solid foam; ^1H NMR (DMSO) δ 1.55−2.5 (m, 8H, 3-CH_2 and 4-CH_2), 3.3−3.8 (m, 4H, 5-CH_2), 3.8 (s, 4H, $COCH_2N$), 4.55 (m, 1H, 2-CH), 4.85 (m, 1H, 2-CH), 7.15 (m, 4H_{arom}), 7.65 (m, 4H_{arom}), 8.35 (br s, 6H, N^+H), 8.5 (br s, 1H, N^+H), 10.35 (br s, 1H, N^+H), 11.0 (s, 2H, NH). Anal. ($C_{25}H_{33}N_6O_6P$·3HCl·$2.5H_2O$) C, H, N.

Bis[4-((*S*)-alanylamino)phenyl] 1-((*S*)-prolyl)pyrrolidine-2*(R,S)*-phosphonate trihydrochloride (11j): B; yield 70%, solid foam; ^1H NMR (D_2O) δ 1.49 (d, 6H, CH_3), 1.65−2.60 (m, 8H, 3-$CH_2$and 4-CH_2), 3.23−3.75 (m, 4H, 5-CH_2), 4.12 (m, 2H, CH of Ala), 7.05 (m, 4H, H_{arom}), 7.35 (m, 4H, H_{arom}). Anal. ($C_{27}H_{37}N_6O_6P$·3HCl·$0.5H_2O$) C, H; N: calcd, 12.16; found, 11.73.

Bis[4-((*S*)-pyroglutamylamino)phenyl] 1-((*S*)-prolyl)-pyrrolidine-2*(R,S)*-phosphonate hydrochloride (11k): B; yield 95%, solid foam; ^1H NMR (DMSO) δ 1.5−2.4 (m, 16H, 3-CH_2 and 4-CH_2), 3.1−4.2 (m, 6H, 5-CH_2 and 5-CH), 4.5 (m, 1H, 2-CH Pro), 4.85 (m, 1H, 2-CH Pro^P), 7.1 (m, 4H_{arom}), 7.65 (m, 4H_{arom}), 7.9 (s, 2H, NH), 8.6 (m, 1H, N^+H), 10.1 (m, 1H, N^+H), 10.3 (s, 2H, NH). Anal. ($C_{31}H_{37}N_6O_8P$·1.5HCl·$3H_2O$) C, H, N.

Bis[4-*(S)*-(2-methoxycarbonyl-2-acetamidoethyl)phenyl] 1-((*S*)-prolyl)pyrrolidine-2-phosphonate hydrochloride (11l): B; yield 85%, solid foam; ^1H NMR (DMSO) δ 1.6−2.6 (m, 8H, 3-CH_2 and 4-CH_2), 1.9 (s, 6H, $COCH_3$), 3.0 (d, 4H, CH_2Ar), 3.2−3.8 (m, 4H, 5-CH_2), 3.7 (s, 6H, $COOCH_3$), 4.4−4.9 (m, 4H, 2-CH), 7.1 (m, 8H_{arom}), 8.2 (d, 2H, AcNH), 8.7 (m, 1H, N^+H), 10.4 (m, 1H, N^+H); MS (FAB^+) *m/z* 687 $(M + H)^+$. Anal. ($C_{33}H_{43}N_4O_{10}P$·1.5HCl·$2H_2O$) C, N; H: calcd, 6.29; found, 5.81.

Bis[4-(methoxycarbonyl)phenyl] 1-((*S*)-prolyl)pyrrolidine-2*(R,S)*-phosphonate hydrochloride (11m): yield 82%, solid foam; ^1H NMR ($CDCl_3$) δ 1.62−2.78 (m, 8H, 3-CH_2 and 4-CH_2), 3.21−3.64 (m, 4H, 5-CH_2), 3.82 (s, 6H, OCH_3), 4.71, 4.90 (m, 2H, 2-CH), 7.21 (m, 4H, H_{arom}), 7.91 (m, 4H, H_{arom}), 8.15 (br, 1H, N^+H), 10.65 (br, 1H, N^+H); MS (FAB^+) *m/z* 517 $(M + H)^+$. Anal. ($C_{25}H_{29}N_2O_8P$·2HCl·$2H_2O$) C, H; N: calcd, 4.48; found, 5.21.

Bis{4-[(ethoxycarbonyl)methylaminocarbonyl]-phenyl} 1-((*S*)-prolyl)pyrrolidine-2*(R,S)*-phosphonate (11n): B; yield 86%, solid foam; ^1H NMR (DMSO) δ 1.3 (t, 6H, CH_3), 1.4−2.6 (m, 8H, 3-CH_2 and 4-CH_2), 3.2−3.8 (m, 4H, 5-CH_2), 4.0 (m, 4H, NCH_2CO), 4.2 (q, 4H, OCH_2), 4.6 (m, 1H, 2-CH), 4.9 (m, 1H, 2-CH), 7.3 (m, 4H_{arom}), 7.9 (m, 4H_{arom}), 8.7 (m, 4H, N^+H and NH); MS (FAB^+) *m/z* 659 $(M + H)^+$. Anal. ($C_{31}H_{39}N_4O_{10}P$·HCl·$1.5H_2O$) C, H, N.

Bis{4-[2-(methoxycarbonyl)ethylaminocarbonyl]-phenyl} 1-((*S*)-prolyl)pyrrolidine-2*(R,S)*-phosphonate hydrochloride (11o): B; yield 79%, solid foam; ^1H NMR (DMSO) δ 1.2−2.8 (m, 8H, 3-CH_2 and 4-CH_2), 2.65 (t, 4H, CH_2COO), 3.2−3.9 (m, 8H, 5-CH_2 and CH_2N), 3.8 (s, 6H, $COOCH_3$), 4.7 (m, 1H, 2-CH), 4.9 (m, 1H, 2-CH), 7.55 (m, 4H_{arom}), 8.15 (m, 4H_{arom}), 8.55 (m, 4H, N^+H and NH).

Bis[4-(*n*-propylaminocarbonyl)phenyl] 1-((*S*)-prolyl)-pyrrolidine-2*(R,S)*-phosphonate hydrochloride (11p): B;

yield 80%, solid foam; ^{1}H NMR (CD_3OD) δ 0.95 (t, 6H, CH_3), 1.6 (m, 4H, CH_2), 1.7−2.6 (m, 8H, 3-CH_2 and 4-CH_2), 3.2−3.9 (m, 8H, 5-CH_2 and CH_2N), 4.65 (m, 1H, 2-CH Pro), 5.1 (m, 1H, 2-CH Pro^P), 7.3 (m, 4H$_{arom}$), 7.85 (m, 4H$_{arom}$); ^{13}C NMR (CD_3OD) δ 11.7 (CH_3), 23.5 (CH_2), 25.0 (4-CH_2), 25.8 (4-CH_2), 27.3 (3-CH_2), 29.7 (3-CH_2), 42.8 (NCH_2), 47.4 (5-CH_2), 48.3 (5-CH_2), 55.9 (d, 2-CH−P, ^1J (C−P) = 161 Hz), 60.6 (2-CH), 121.4, 130.4, 133.1, 153.7 (C$_{arom}$), 168.8 (CO), 169.2 (CO).

2,2′-Biphenylyl Acetyl Phosphite (16). A solution of chloroanydride **15** (0.152 mol, 38 g) in dry THF (50 mL) was added dropwise to a solution of AcOH (0.152 mol, 9.1 mL) and Et_3N (0.152 mol, 21.3 mL) in dry THF (100 mL) with stirring at 0 °C. The cooling was removed, and the mixture was stirred at room temperature for 3 h. The solid was filtered off, and the filtrate was evaporated. The crude product was used in the next step without further purification: yield 40 g (96%), viscous oil; ^{1}H NMR ($CDCl_3$) δ 2.1 (s, 3H, CH_3CO), 6.9−7.5 (m, 8H, H$_{arom}$).

2,2′-Biphenylyl 1-(benzyloxycarbonyl-*(S)*-prolyl)pyrrolidine-2*(R,S)*-phosphonate (17a): prepared according to the general procedure for diaryl phosphonates with acetal **6a** and phosphite **16**; yield 27%, solid foam; ^{1}H NMR ($CDCl_3$) δ 1.2−2.9 (m, 8H, 3-CH_2 and 4-CH_2), 3.1−4.0 (m, 4H, 5-CH_2), 4.55 (m, 1H, 2-CH Pro), 4.85 (m, 1H, 2-CH Pro^P), 5.2 (br s, 2H, CH_2Ph), 7.1−7.85 (m, 15H$_{arom}$); MS (EI) *m/z* (relative intensity) 532 (M, 40), 397 (20), 300 (26), 232 (29), 215 (41), 204 (45), 168 (42), 160 (74), 91 (100), 70 (48).

2,2′-Biphenylyl 1-(*tert*-butyloxycarbonyl-*(S)*-prolyl)-pyrrolidine-2*(R,S)*-phosphonate (17b): prepared according to the general procedure for diaryl phosphonates with acetal **6b** and phosphite **16**; yield 24%, solid foam; ^{1}H NMR ($CDCl_3$) δ 1.3−2.55 (m, 8H, 3-CH_2 and 4-CH_2), 1.5 (s, 9H, CH_3), 3.35−3.9 (m, 4H, 5-CH_2), 4.55 (m, 1H, 2-CH), 4.9 (m, 1H, 2-CH), 6.95−7.75 (m, 8H, H$_{arom}$); MS (FAB^+) *m/z* 499 $(M + H)^+$.

2-(2′-Hydroxyphenyl)phenyl methyl 1-(benzyloxycarbonyl-*(S)*-prolyl)pyrrolidine-2*(R,S)*-phosphonate hydrochloride (18): prepared from the Z-protected derivative **17a** using procedure B for diaryl phosphonates; yield 92%, solid foam; ^{1}H NMR ($CDCl_3$) δ 1.5−2.6 (m, 8H, 3-CH_2 and 4-CH_2), 3.1−3.9 (m, 7H, 5-CH_2 and OCH_3), 4.4−4.8 (m, 2H, 2-CH Pro and 2-CH Pro^P), 6.85 (br s, 1H, PhOH), 7.1−7.6 (m, 8H$_{arom}$), 8.4 (m, 1H, N^+H), 9.6 (m, 1H, N^+H); ^{13}C NMR ($CDCl_3$) δ 25.7 (4-CH_2), 26.1 (4-CH_2), 28.2 (3-CH_2), 30.5 (3-CH_2), 48.1 (5-CH_2), 48.6 (5-CH_2), 55.6 (OCH_3), 55.6 (2-CH−P, ^1J (C−P) = 142 Hz), 60.8 (2-CH), 117.8, 121.0, 126.3, 130.1, 132.5, 133.8 149.7, 156.2 (C$_{arom}$), 168.9 (CO); MS (FAB^+) *m/z* 431 $(M + H)^+$. Anal. ($C_{22}H_{27}N_2O_5P\cdot HCl\cdot H_2O$) C, H; N: calcd, 5.78; found, 6.26.

2,2′-Biphenylyl 1-((*S*)-prolyl)pyrrolidine-2*(R,S)*-phosphonate hydrochloride (19): prepared from the Boc-protected derivative **17b** using procedure A for diaryl phosphonates; yield 81%, solid foam; ^{1}H NMR ($CDCl_3$) δ 1.3−2.55 (m, 8H, 3-CH_2 and 4-CH_2), 3.35−3.75 (m, 4H, 5-CH_2), 4.75 (m, 2H, 2-CH), 7.0−7.5 (m, 8H, H$_{arom}$), 10.9 (m, 2H, N^+H); ^{13}C NMR ($CDCl_3$) δ 24.4 (4-CH_2), 24.9 (4-CH_2), 26.7 (3-CH_2), 28.7 (3-CH_2), 46.6 (5-CH_2), 51.9 (5-CH_2), 53.4 (2-CH−P, ^1J (C−P) = 151 Hz), 59.2 (2-CH), 116.9, 120.9, 121.7, 121.9, 125.8, 126.6, 128.4, 129.0, 130.3, 131.5, 147.9 (C$_{arom}$), 167.8 (CO); MS (FAB^+) *m/z* 399 $(M + H)^+$. Anal. ($C_{21}H_{23}N_2O_4P\cdot 1.4HCl\cdot 1.1H_2O$) C, H; N: calcd, 5.97; found, 5.43.

Bis(2,2,2-trichloroethyl) 1-(benzyloxycarbonyl-*(S)*-prolyl)pyrrolidine-2*(R,S)*-phosphonate (20): Prepared according to the general procedure for diaryl phosphonates with acetal **6a** and tris(2,2,2-trichloroethyl) phosphite; yield 31%, oil; ^{1}H NMR ($CDCl_3$) δ 1.2−2.8 (m, 8H, 3-CH_2 and 4-CH_2), 3.3−4.1 (m, 4H, 5-CH_2), 4.3−5.1 (m, 6H, CH_2CCl_3 and 2-CH), 5.2 (br s, 2H, CH_2Ph), 7.35 (s, 5H, H$_{arom}$); MS (FAB^+) *m/z* 645 $(M + H)^+$.

Bis(2,2,2-trifluoroethyl) 1-(benzyloxycarbonyl-*(S)*-prolyl)pyrrolidine-2*(R,S)*-phosphonate (21): prepared according to the general procedure for diaryl phosphonates with acetal **6a** and tris(2,2,2-trifluoroethyl) phosphite; yield 48%, oil; ^{1}H NMR ($CDCl_3$) δ 1.2−2.7 (m, 8H, 3-CH_2 and 4-CH_2), 3.2−4.1 (m, 4H, 5-CH_2), 4.2−5.0 (m, 6H, CH_2CF_3 and 2-CH), 5.2 (br s, 2H, CH_2Ph), 7.35 (s, 5H, H$_{arom}$); MS (FAB^+) *m/z* 546 $(M + H)^+$.

Bis(2,2,2-trichloroethyl) 1-((*S*)-prolyl)pyrrolidine-2*(R,S)*-phosphonate hydrochloride (22): prepared from the Z-protected derivative **20** using procedure B for diaryl phosphonates; yield 26%, solid foam; ^{1}H NMR ($CDCl_3$) δ 1.5−3.0 (m, 8H, 3-CH_2 and 4-CH_2), 3.2−4.1 (m, 4H, 5-CH_2), 4.4−5.1 (m, 6H, CH_2CCl_3 and 2-CH), 6.55 (m, 1H, N^+H), 8.4 (m, 1H, N^+H); MS (FAB^+) *m/z* 511 $(M + H)^+$.

Bis(2,2,2-trifluoroethyl) 1-((*S*)-prolyl)pyrrolidine-2*(R,S)*-phosphonate hydrochloride (23): prepared from the Z-protected derivative **21** using procedure B for diaryl phosphonates; yield 44%, solid foam; ^{1}H NMR ($CDCl_3$) δ 1.3−2.8 (m, 8H, 3-CH_2 and 4-CH_2), 3.2−4.0 (m, 4H, 5-CH_2), 4.4−5.1 (m, 6H, CH_2CF_3 and 2-CH), 6.9 (m, 1H, N^+H), 8.4 (m, 1H, N^+H); MS (FAB^+) *m/z* 413 $(M + H)^+$. Anal. ($C_{13}H_{19}F_6N_2O_4P\cdot HCl\cdot 0.8H_2O$) C; H: calcd, 4.70; found, 5.51. N: calcd, 6.05; found, 6.72.

DPP IV Inhibition in Vitro and Stability Measurements. DPP IV was purified from human seminal plasma as described previously.[32] Enzymatic activity was measured at 37 °C in a Spectramax 340 (Molecular Devices) microtiter plate reader using Gly-Pro-*p*-nitroanilide (Sigma) as a chromogenic substrate. The reaction was monitored at 405 nm, and the initial rate was determined between 0 and 0.25 absorbance units. The reaction mixture contained 2 mM substrate, approximately 1 mU of DPP IV, 40 mM TRIS-HCl buffer, pH 8.3, and a suitable amount of inhibitor (ranging between 0 and 10 mM) in a total volume of 0.2 mL. Activity measurements were routinely performed in duplicate. The IC_{50} value is defined as the concentration of inhibitor required to reduce the DPP IV activity to 50% after a 15-min preincubation with the enzyme at 37 °C before addition of the substrate. Inhibitor stock solutions (100 mM) were prepared in DMSO or phosphate buffer, pH 7.4, depending on the solubility of the compound and stored at −20 °C. Stock solutions were diluted with 50 mM TRIS-HCl buffer, pH 8.3, as required immediately before the experiment. Since the compounds described in this paper completely inactivate DPP IV following pseudo-first-order kinetics, the IC_{50} value is inversely correlated with the second-order rate constant of inactivation.[11] For a simple pseudo-first-order inactivation process, the activity after incubation with inhibitor (v_i) varies with the inhibitor concentration (i) as described in the following equation: $v_i = v_o \times e^{-kit}$, where v_o is the activity in absence of inhibitor, k is the second-order rate constant of inactivation, and t is the time. Since at $i = IC_{50}$ by definition $v_i = {}^1/_2 v_o$, it follows from the equation that $k = \ln 2/(t_i \times IC_{50})$, where $t_i = 15$ min. These calculated k values are listed in Table 1. For some inhibitors (**5, 11a, 11b, 11d, 11e, 11h, 11n**) the inactivation rate constant was determined from the time course of inhibition as described before.[11]

The functional stability of the inhibitors was estimated by measuring the inhibitory potency (apparent IC_{50}) of a 1 mM dilution of the compounds in citrated human plasma at three or four time points between 0 and 300 min at 37 °C. Fitting the inverse of the apparent IC_{50} values versus time with a single-exponential decay gives the half-lives reported in Table 1.

In Vitro Cytotoxicity and Efficacy in Human Peripheral Blood Mononuclear Cells (PBMC). Peripheral blood mononuclear cells were isolated from buffy coats (obtained from the blood transfusion center of Antwerp). After dilution (1/4) in phosphate buffer saline (PBS), cells were layered onto Ficoll-Hypaque density gradient (Pharmacia, Uppsala, Sweden) and centrifuged at room temperature at 550*g* during 20 min. The interfaces were collected and washed three times in RPMI-1640. Finally the cells were resuspended at 1×10^6 cells/mL in RPMI-1640 containing 10% heat-inactivated fetal calf serum and antibiotics (penicillin/streptomycin) (Gibco). These freshly isolated cells were used immediately for inhibitor studies or first stimulated with phytohemagglutinin (Murex diagnostics) at 1 μg/mL during 3 days at 37 °C in a 5% CO_2

humidified incubator. Inhibitor (stock solution at −80 °C in phosphate buffer, diluted ex tempore in RPMI-1640) or vehicle alone was added to the cells (5×10^6/test) at different concentrations. After overnight incubation at 37 °C, an aliquot was taken for cytotoxicity evaluation by 0.4% trypan blue exclusion. The remaining cells were washed three times in PBS, and the final cell pellet was solubilized in 200 μL of PBS containing 1% v/v Triton X-100 and 100 KIU/mL aprotinin (Bayer) and centrifuged during 10 min at 20000*g*. Supernatants were used immediately for enzyme assay and protein determination by the Bradford microassay. Specific activities (U/g protein) are compared, and the percent inhibition is given toward control samples without inhibitor.

DDP IV Inhibition in Vivo. Male New Zealand white rabbits (2.5−3.5 kg), Wistar rats (250−350 g), and Swiss mice (23−36 g) were allowed to adjust to their environment for at least 7 days. They received standard diet and water ad lib. Test compounds were dissolved in 50 mM phosphate buffer, pH 7.4, at concentrations ranging from 10 to 100 mg/mL, stored in aliquots at −80 °C, and thawed ex tempore. Rabbits received a single slow intravenous bolus injection of test compound or vehicle alone in the marginal ear vein. Blood was sampled from the central ear artery. Rats and mice were injected subcutaneously or intraperitoneally. Rat blood samples were obtained under anesthesia (Forene) from the vena femoralis by puncture after incision of the skin. The mice were bled by orbita puncture after induction of anesthesia with pentobarbital. After clotting, blood samples were centrifuged (3000*g*, 10 min), and the resulting sera were stored at −80 °C until assayed for DPP IV activity.

Determination of Specificity for DPP IV. Prolyl endopeptidase and elastase activity were determined as previously described.[33,34] Dipeptidyl peptidase II activity was determined by the hydrolysis of Lys-Ala-4-MeO-2-NA (1.4 mM) (Sigma, L-2270) in 100 mM acetate buffer, pH 5.5, containing 2 mM EDTA. Hydrolysis of L-Ala-4-MeO-2-NA (5 mM) (Sigma, A-5414) in 60 mM phosphate buffer (pH 7.4) was chosen for the determination of membrane alanyl aminopeptidase activity.

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