Structure−Activity Relationships of Small Phosphopeptides, Inhibitors of Grb2 SH2 Domain, and Their Prodrugs

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To develop potential antitumor agents directed toward HER2/ErbB2 overexpression in cancer, we have designed inhibitors of the recognition between the phosphotyrosine of the receptor and the SH2 domain of the adaptor protein Grb2. In the first part of the paper, we report the synthesis of mimetics of the constrained (α-Me)phosphotyrosine residue such as (α-Me)-4-phosphonomethylphenylalanine ($-CH_2PO_3H_2$), (α-Me) 4-phosphonodifluoromethylphenylalanine ($-CF_2PO_3H_2$), and (α-Me)-4-phosphonophenylalanine ($-PO_3H_2$). The incorporation of these residues in the *m*AZ-pTyr-Xaa-Asn-NH_2 series provided compounds with very high affinity for the Grb2 SH2 domain, in the $10^{-8}-10^{-9}$ range of K_d values. These compounds behave as potent antagonists of the Grb2−Shc interaction. Our results highlight the importance of the doubly negative charge borne by the pY + 1 amino acid in accordance with the interactions observed in the complex crystallized between *m*AZ-pTyr-(αMe)pTyr-Asn-NH_2 and the Grb2 SH2 domain. *m*AZ-pTyr-(αMe)pTyr-Asn-NH_2 was derivatized as the *S*-acetyl thioester (SATE) of the phosphotyrosine residues, and its surrogates provided prodrugs with very potent antiproliferative activity on cells overexpressing HER2/ErbB2, with ED_{50} values amounting to 0.1 μM. Finally a new prodrug is put forth under the form of a monobenzyl ester of phosphate group that is as active as and much easier to synthesize than SATE prodrugs. These compounds show promising activity for further testing on in vivo models.

Introduction

Cellular proliferation and differentiation are regulated by a variety of signaling mitogens such as growth factors that bind to the extracellular domain of their receptors. This process induces receptor dimerization and trans-phosphorylation of several intracellular tyrosine residues in its C-terminal part and results in protein recruitment and transduction of the growth factor signal inside the cell. Deregulation of the Ras signaling pathway has been involved in a number of diseases that include leukemia and several cancers.[2] Along this pathway, the small adaptor protein Grb2 (growth factor receptor-bound protein 2) constitutes a connector between the receptor and Sos, the exchange factor of Ras. Grb2 is composed of a single SH2 (Src homology) domain flanked by two SH3 domains.[3] Grb2 SH2 domain binds numerous tyrosine phosphorylated proteins including activated RTKs such as the members of erbB family,[4] docking proteins such as Shc,[5] and cytoplasmic tyrosine kinases such as Bcr-Abl.[6] It was shown that direct binding of Grb2 through its SH2 domain with the Bcr-Abl is required for efficient induction of chronic leukemia-like diseases in mice.[7] Grb2 forms a complex through its SH3 domains with Sos, which in turn activates Ras by exchanging its GDP binding form to the GTP binding one. Because of the Grb2 role in the Ras signaling pathway and its upregulation in human breast cancer[8] and human bladder cancer[9] and in the early events of mice liver carcinogenesis,[10] inhibition of Grb2 constitutes an attractive strategy for developing new antitumor agents.[11,12]

Since the Grb2 SH2 domain recognizes with high affinity and specificity the phosphotyrosyl consensus motif -pY-X-N- (pY, phosphotyrosine; X, any hydrophobic amino acid; N, asparagine) on its targets,[13] the development of SH2 domain inhibitors was carried out following several directions. The first is the search for peptide inhibitors, encompassing phosphonate and carboxylate-based pY mimetics that are resistant to intracellular phosphatases.[14] The second consists of modifying the structure of peptide inhibitors to circumvent the lack of cell permeability that is due to the presence of negatively charged groups.[15−18] The third consists of the design of peptidomimetics and even nonpeptidic compounds to inhibit Grb2−SH2 interactions.[19]

These directions of research include (i) the optimization of the N- and C-terminal groups and of the modified hydrophobic residue X of the minimum pY-X-N peptide,[20−23] (ii) the design of phosphorylated as well as unphosphorylated cyclic peptides,[25,26] and (iii) the search for peptidomimetics retaining little or no peptidic character.[27−31]

In a previous paper,[22] we reported the rational design and synthesis of derivatives in the *m*AZ-pTyr-Xaa-Asn-NH_2 series, which had high affinity for Grb2 especially when Xaa is an (α-Me)pTyr residue. The lower affinities of the peptides containing carboxylate mimetics of the (α-Me)pTyr residue confirmed that the doubly nega-

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Table 1. [a]

compound	peptide sequence	K_d (nM)[b]	IC_{50} (nM)[c]
P1	*m*AZ-pTyr-(L)(α-Me)pTyr-Asn-NH_2*	3 ± 1	11 ± 1
P2	*m*AZ-pTyr-(L)(α-Me)Phe(CH_2CO_2H)-Asn-NH_2*	60 ± 10	198 ± 41
P3	*m*AZ-pTyr-(L)(α-Me)Phe(CO_2H)-Asn-NH_2*	45 ± 10	153 ± 38
P4	*m*AZ-pTyr-(D,L)(α-Me)Pmp-Asn-NH_2	70 ± 30	265 ± 35
P5	*m*AZ-pTyr-(D,L)(α-Me)F_2Pmp-Asn-NH_2	nm	64 ± 11
P6	*m*AZ-pTyr-(L)(α-Me)Ppp-Asn-NH_2	4.5 ± 4.2	14 ± 2
P7	*m*AZ-pTyr-(D)(α-Me)Ppp-Asn-NH_2	nm	113 ± 21
P8	*m*AZ-Pmp-(α-Me)pTyr-Asn-NH_2	17 ± 8	42 ± 22
P9	*m*AZ-Pmp-(D,L)(α-Me)Pmp-Asn-NH_2	835 ± 258	nd

[a] The asterisk (*) represents reference peptides.[22] nm: non measurable. nd: not determined. [b] K_d values were measured by fluorescence[22] (±SD). [c] IC_{50} values were determined by ELISA[22] (±SD).

tively charged phosphate of the residue at position pY + 1 is very important for the interactions with the Grb2 SH2 domain[22] (see Table 1). Here, we describe the synthesis of three phosphonate mimetics of (α-Me)pTyr in the search for better phosphatase-resistant analogues and their further incorporation into the peptide sequence.

Because these inhibitors did not enter cells, they were modified under the form of cell-penetrating prodrugs. We have thus derived esterase-sensitive prodrugs of phosphotyrosine residue. The first two, **P10** and **P11**, are *S*-acyl thioethylester (SATE) prodrugs of the phosphate groups in the diphosphorylated peptide **P1**. The third one, **P12**, is a benzyl ester prodrug of the phosphogroups introduced into Ac-pY-(α-Me)pY-N-NH-$(CH_2)_3$-(1-naphthyl). Such prodrugs can decompose and release the active agents in the cells so that their antiproliferative effects could be tested.

Design

Cα-Methylated Phosphotyrosine Mimetics: Synthesis and Incorporation in the Peptide Sequences. In an earlier work, we had incorporated as Xaa two carboxylate mimetics of (α-Me)pTyr in the *m*AZ-pTyr-Xaa-Asn-NH_2 series,[22] namely, **P2** and **P3** reported in Table 1, that showed lower binding affinity for Grb2 when compared to the diphosphorylated peptide **1** (**P1**). Such a decreased affinity was suggested to be related to the single charge of the carboxyl group borne by the aromatic ring that could not provide as many interactions with the SH2 domain as dicharged phosphates. In accordance, molecular modeling had earlier shown that in the diphosphorylated **P1** peptide, the (α-Me)pTyr amino acid can form additional interactions with the side chain residues W^{121}, S^{141}, R^{142}, and N^{143} of the Grb2 SH2 domain. Such a prediction was subsequently confirmed by the high-resolution X-ray structure of the complex of this peptide with the Grb2 SH2 domain.[32]

In the present paper, we report the synthesis of Cα-methylated phenylalanine bearing in the para position of the aromatic ring phosphonomimetics such as CH_2-PO_3H_2 (α-Me)Pmp, CF_2-PO_3H_2 (α-Me)F_2Pmp, and PO_3H_2 (α-Me)Ppp groups designed to provide doubly negative charge at physiological pH (Figure 1a). In the case of para-substituted Phe without Cα-Me substitution, it was known that the CH_2-phosphonate group is not completely ionized at the physiological pH, and thus, peptides including it generally have weakened interactions with SH2 domains.[33,34] Such a mimetic is the simplest to synthesize, and in some cases its substitution provides compounds with similar affinities as the

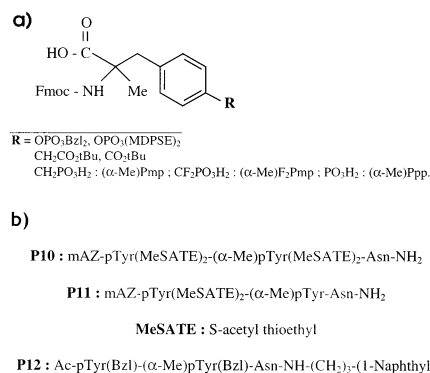


Figure 1. (a) Mimetics of pTyr protected for solid-phase peptide synthesis. (b) Prodrug formula.

phosphate analogue.[35] Burke et al. have developed the 4-CF_2-phosphonate of phenylalanine,[36] which has a pK_a similar to that of pTyr[37] and showed that some peptides including this surrogate have even better affinities than the original ones.[38] We have also designed in the present study a novel amino acid, (α-Me) p(PO_3H_2)-Phe with a para PO_3H_2 phosphonate group directly attached to the aromatic ring. Such a phosphonate group is more acidic than the CH_2-phosphonate one and is also more stable and easier to synthesize than the CF_2-phosphonate. Its non-α-methylated analogues had already been used to synthesize highly potent inhibitors of the Src SH2 domain.[39,40]

Prodrugs. Enzyme-labile modifications of the phosphate group such as esters or phosphoramidate derivatives have been reviewed.[41,42] We have previously shown that the MeSATE is an appropriate protecting group for the cellular transport of phosphopeptides,[18] which act as inhibitors of the Grb2 SH2 domain. On the basis of such results, we now report the design of two prodrugs of the diphosphorylated, most active peptide **P1** (Figure 1b). In the first prodrug, denoted as the totally protected prodrug (**P10**), both phosphate groups were derivatized with the di-MeSATE phosphate protections. Since this compound had a very low water solubility, a second compound (**P11**) was synthesized in which only one phosphate group was derivatized.

Although less efficient than the SATE analogues, benzyl esters were found to have prodrug protector properties of the phosphinate group.[43] These results led us to investigate benzyl-protected phosphopeptides as prodrugs. An assay on a monophosphotyrosine-containing peptide derived from the Shc 317 sequence (Ac-PFpYVNVP−NH_2), and with such a benzyl phosphate protection, showed this peptide to have similar cellular antiproliferative activity (unpublished results) as its

MeSATE analogue.[18] This result prompted us to design the **P12** prodrug (Figure 1b) in which we took into account the following experimental findings: (i) the results from the high-resolution X-ray data showing that the *m*AZ group of **P1**, essential in the case of Novartis group peptides, is less important in our series and might be replaced by an acetyl group without greatly affecting the affinity;[32] (ii) the finding by Burke et al. showing that a C-terminal (1-naphthyl)propyl group increased the cellular permeability of phosphonopeptides even with a free phosphonate group;[35] (iii) the poor water solubility of the di-SATE phosphopeptide **P10**. We have thus designed and synthesized peptide **P12** (Ac-pY-(α-Me)pY-N-NH-$(CH_2)_3$-(1-naphthyl)), which was derivatized as the monobenzyl ester of each phosphate group. Such a compound is chemically more accessible than the corresponding monodibenzylphosphopeptide.

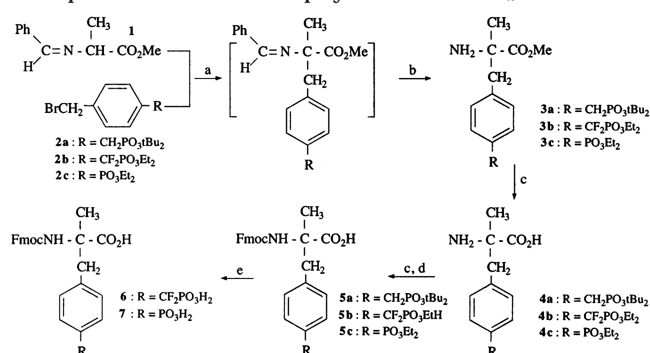
Synthesis

Synthesis of (α-Me) pTyr Mimetics. Preparation of (α-Me)pTyr with dibenzyl phosphoester protection for use in solid-phase peptide synthesis (SPPS) through Fmoc chemistry was already described.[22] In the present paper, the monobenzyl-protected phosphate of Fmoc-(α-Me)pTyr, useful for the synthesis of **P12**, was prepared by refluxing the dibenzyl analogue with NaI in acetonitrile as reported for the preparation of monobenzyl-protected pSer or pThr analogues.[44] The phosphate group was also diprotected as MDPSE ((methyldiphenylsilyl)ethyl), since this group is a more stable and appropriate protection group for the SPPS.[45]

During the synthesis of phosphatase-resistant mimetics of (α-Me)pTyr, we have attempted to do an enantioselective synthesis of the phosphonate analogues, following the method of Williams that we previously used for the preparation of the two carboxylate mimetics.[22] This has, however, remained unsuccessful. Therefore, the phosphonate analogues were prepared under racemic forms and expected to be separated in the corresponding peptides.

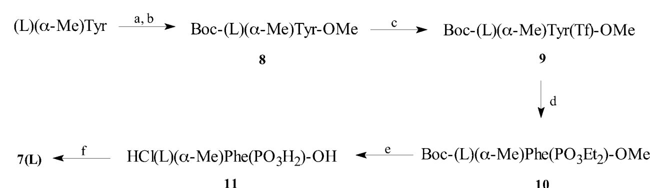
The method previously described for the synthesis of Pmp was applied to the sterically constrained analogue (α-Me)Pmp.[46] The Fmoc-protected (α-Me)Pmp, (α-Me)-F_2Pmp, and (α-Me)Ppp (Ppp: 4-phosphonyl-Phe) were prepared by phase-transfer catalyzed alkylation of alanine benzylidene methyl ester **1** with para-substituted benzyl bromides (see Scheme 1). Compound **2a** was prepared according to a previously reported method.[46] **2b** was prepared by fluorinating $CH_3-C_6H_4-CH_2-PO_3Et_2$ by NFBS[47] followed by NBS benzyl bromination. **2c** was prepared by substitution of 4-bromotoluene with triethyl phosphite catalyzed by Pd^0 [48] followed by NBS bromination. The products of alkylation were directly hydrolyzed with 5% citric acid to give compounds **3** (**3a**, **3b**, and **3c**). Saponification of compounds **3** gave free amino acids **4** that were protected with Fmoc-Cl under basic conditions (pH 9). Because of the steric hindrance introduced by the α-methyl group, the yield of the Fmoc protection was relatively low (35−65%). The Fmoc protected compound **5a** as the *t*Bu phosphonate ester was suitable for solid-phase peptide synthesis in Fmoc chemistry. Nevertheless, such a *t*Bu protection was not possible for the CF_2-phosphonate and phosphonate because of instability or steric hindrance. Thus, the last two phosphonates were derived as ethyl esters, which had to be removed by a TMSI treatment before synthesis of the peptide[49] to give compounds **6** and **7**, since such a treatment might hydrolyze the N-terminal *m*AZ group of the *m*AZ-pTyr-Xaa(Et_2)-Asn-NH_2 peptide and could not provide the expected peptide.

Scheme 1. Synthesis of (α-Me) pTyr Surrogates: (α-Me)p$CH_2PO_3H_2$-Phe, (α-Me)p$CF_2PO_3H_2$-Phe, and (α-Me)pPO_3H_2-Phe as Fmoc for Peptide Synthesis and Incorporation in the *m*AZ-pTyr-Xaa-Asn-NH_2 Series[a]



[a] Reagents: (a) $KOH/K_2CO_3/Et_3PhCH_2N^+Cl^-/CH_2Cl_2$; (b) 5% citric acid; (c) 1 N NaOH; (d) CO_2, Fmoc-Cl; (e) TMSI/CH_3CN.

Scheme 2[a]



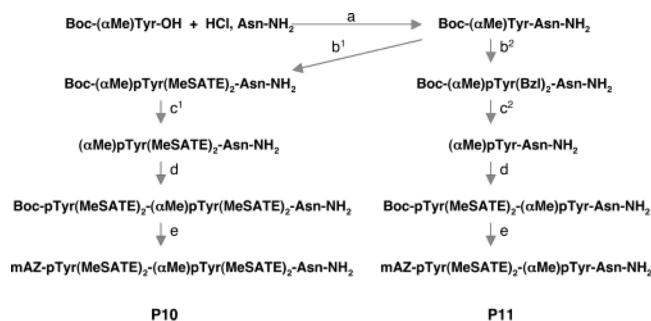
[a] Reagents: (a) $SOCl_2$/MeOH; (b) $Boc_2O/NaHCO_3$; (c) $(CF_3SO_2)_2N$-Ph, Et_2N; (d) HPO_3Et_2, $Pd(PPh_3)_4$, Et_3N; (e) 9 N HCl, reflux; (f) Fmoc-Cl.

Alternatively, we have also prepared the (α-Me)Ppp **7** as the (L)-form by substitution of *O*-triflate of (L)(α-Me)Tyr (see Scheme 2). (L)(α-Me)Tyr was first esterified by refluxing in $SOCl_2$/MeOH. The methyl ester obtained was then protected as *N*-Boc to obtain compound **8**. Protection was much more difficult to obtain than usually probably because of the steric hindrance. An excess of Boc_2O and longer reaction times were necessary for a complete reaction. Moreover, the reaction mixture should be maintained at a pH of less than 9; otherwise, the phenolic group would also be protected as Boc. The phenolic group of Boc-(L)(α-Me)Tyr-OMe was then transformed into triflate ester **9** and substituted by diethyl phosphite with tetrakis(triphenylphosphine)palladium as catalyst[16] (compound **10**). All the protections were then removed to give compound **11** by refluxing the Boc-(L)(α-Me)Phe(4-PO_3Et_2)-OMe in 9 N HCl overnight. The free amino acid was reprotected as Fmoc-(L)(α-Me)Phe(4-PO_3Et_2), **7**(L), by Fmoc-Cl.

Finally, these mimetics of (α-Me)pTyr were used for coupling with HATU/HOAt in order to prepare the peptide sequence *m*AZ-pTyr-Xaa-Asn-NH_2 (Xaa: (α-Me)-pTyr mimetics) by solid-phase peptide synthesis following the procedure already described.[22]

Prodrugs. A small scale of the totally protected prodrug (**P10**) was first synthesized in SPPS. The necessary MeSATE protected pTyr and (α-Me)pTyr components were prepared as already described.[50,18] The *m*AZ motif was prepared but in low yield as the Fmoc-

Scheme 3[a]



[a] Reagents: (a) HATU/HOAt, DIEA; (b^1) (1), $Et_2NP(OMeSATE)_2$, tetrazole; (2) *t*BuOOH; (b^2) (1) *i*$Pr_2NP(OBzl)_2$, tetrazole (2), *t*BuOOH; (c^1) 30% TFA in CH_2Cl_2; (c^2) TFA; (d) Boc-pTyr(MeSATE)$_2$-OH/HATU/HOAt/DIEA; (e) (1) 30% TFA in CH_2Cl_2; (2) Boc-*m*AZ-ONp-DIEA; (3) 30% TFA in CH_2Cl_2.

*m*AZ-ONp form. This compound is much more unstable than its Boc-protected analogue.

Peptide synthesis was carried on a Siber amide resin with HATU/HOAt coupling and 2% DBU Fmoc deprotection because the classical 20% piperidine Fmoc deprotection condition had been shown to hydrolyze the MeSATE groups as well.[50] The final product was thus obtained after the cleavage of the resin with 5% TFA in CH_2Cl_2.

At the same time, we have also prepared on a larger scale peptide **P10** through solution-phase and Boc chemistry (Scheme 3). The Boc-(α-Me)Tyr was coupled with Asn-NH_2. Because of the high hydrophilicity of the Asn-NH_2 component, the coupling was quite inefficient even using HATU/HOAt as coupling agents. The dipeptide was also hydrophilic and could not be taken up in organic solvent by classical workup and was obtained by lyophilization of the aqueous phase. After phosphorylation with the MeSATE phosphoramidite, 30% TFA was used to remove the Boc group without affecting the MeSATE protection. To avoid concentrating TFA, which might deprotect the MeSATE groups, the reaction solution was coevaporated with cyclohexane to dryness. Repetition of coupling/deprotection led to the final totally protected prodrug.

For the synthesis of the mono-MeSATE-protected prodrug **P11**, the Boc-(α-Me)Tyr-Asn-NH_2 was phosphorylated with benzyl phosphoramidite. Neat TFA was used to completely remove all the protections. Coupling of the deprotected phosphodipeptide (α-Me)pTyr-Asn-NH_2 with Boc-pTyr(MeSATE)$_2$-OH is very unfavorable and was obtained with very low yield (3.6%).

The dimonobenzyl prodrug **P12** was synthesized in the solid phase. Fmoc-Asp-NH-$(CH_2)_3$-(1-naphthyl), prepared by the coupling of Fmoc-Asp(*t*Bu)-OH and 3-(1-naphthyl)-1-propylamine[21] followed by the TFA deprotection of the *t*Bu group, was attached to the predeprotected Siber amide resin. The Fmoc-(α-Me)pTyr(Bzl)-OH and the Fmoc-pTyr(Bzl)-OH were then sucessively introduced with HATU/HOAt/DIEA as coupling agent and piperidine Fmoc deprotection. A capping of the NH_2 terminal of the peptide with acetic anhydride was made before resin cleavage with 2% of TFA in dichloromethane.

For the synthesis of Fmoc(α-Me)-pTyr(Bzl)-OH, L(α-Me)Tyr-OH was first protected as Fmoc on the amino group and the dibenzyl phosphate was introduced on the phenol function using dibenzyl *N*,*N*-dibenzylphosphoramidite and terbutyl hydroperoxyde.[51] Successive NaI treatment of the dibenzyl phosphoesters provided the monobenzyl ester.

Results and Discussion

***m*AZ-pTyr-Xaa-Asn-NH_2 Peptide Affinities for Grb2.** As in our previous paper,[22] the affinities (expressed as dissociation constant K_d) of *m*AZ-pTyr-Xaa-Asn-NH_2 for Grb2 were measured through fluorescence modifications of Grb2 emission spectrum by addition of increasing peptide concentrations. When no fluorescence modifications were observed, IC_{50} values were measured by an ELISA competition test between Grb2 and a phosphopeptide.[22] The results are reported in Table 1 and compared to those of peptides **P1**, **P2**, and **P3** as references.

Because the diastereoisomers of peptides **P4** and **P5** could not be separated by HPLC, their affinity was measured under the form of diastereoisomer mixtures.

Peptide **P4**, containing the racemic (α-Me)Pmp, showed 20-fold lower affinity than the original phosphate peptide **P1** in the same way as the (L)-form of carboxylate monocharged mimetics **P2** and **P3**. This result is due in part to the racemic form of **P4** and in part to the incomplete ionization of the methyl phosphonate group at physiological pH, as had been suggested for several Pmp-containing peptides.[33] Our molecular modeling and X-ray data[22,32] have shown the onset of several hydrogen bonds involving the (α-Me)pTyr phosphate group and hydrogen-bond donors in the SH2 domain of Grb2, namely, R142, N141, and S140. This shows a doubly charged acid group on the pY + 1 residue to be an important determinant of the peptide−SH2 domain interaction.

The **P5** peptide contains the difluoro analogue CF_2-phosphonate, which had been demonstrated to favor the ionization of the phosphonate group. It was not possible to measure its Grb2 binding affinity by fluorescence because only a very slight variation of fluorescence was observed upon addition of the peptide to a Grb2 solution. However, the competition assay showed a 3- to 4-fold higher Grb2 binding capacity than **P4**. The lower affinity of **P5** for Grb2 compared to **P1** originates from the existence of **P5** under the form of a racemic mixture and possibly also from the absence of the phenolic oxygen that may form hydrogen bonds in **P1**. It could also be due to the slightly larger size of the difluoromethylene group than oxygen, since this may cause steric hindrance.

Comparison of the binding affinities of peptides **P2** and **P3** containing a methyl carboxylate substitution or a direct carboxylate on the phenyl ring showed that shortening the acid-bearing side chain improved the peptide affinity. We have accordingly prepared Fmoc-(D,L)(α-Me)Phe(PO_3H_2) and incorporated it into the reference peptide sequence. The expected pK_{a2} of the phosphonate group is lower when the group is attached directly to the phenyl ring than if a CH_2 group is interposed.

The two diastereoisomers of the peptide have been separated by semipreparative HPLC. The absolute configurations of the phosphonate mimetics were confirmed by the synthesis of the (L)-form mimetic and its

peptide. Comparison of their NMR spectra also showed that **P6** belongs to the (L)-form and **P7** to the (D)-form. The singlet signal (δ ppm) of the NH of the (L)-form of the Cα-methylated residue in such a peptide sequence always appears around 8.3, which is the case with **P6**. **P7** has a singlet signal at 8.5 and is attributed to the (D)-isomer.

The affinity of peptide **P6** for Grb2 is very close to that of peptide **P1**, while peptide **P7** with an inversion of the (α-Me)Ppp configuration shows about a 10-fold lower affinity. These results confirm our observation in the series of carboxylate mimetics that a shorter side chain acidic function on the phenyl ring may better contribute to interactions with the SH2 domain of Grb2. Moreover, such results showed that the configuration of the residue at pY + 1 is not very critical for the SH2–Grb2 binding. Therefore, we can deduce that the (L)-form of mimetic peptides **P4** and **P5** will have higher affinity for Grb2 than their carboxylate analogues **P2** and **P3**.

Peptides **P8** and **P9** that have a Pmp group instead of a pTyr one have lower Grb2 binding affinities than **P1**. **P8** undergoes a 5- to 6-fold loss in affinity, whereas peptide **P9**, which bears an (α-Me)Pmp in place of (α-Me)p-Tyr, has an at least 200-fold lower affinity than peptide **P1** and a 10-fold lower affinity than peptide **P4**, which has a (α-Me)Pmp moiety. These results show that the presence of two doubly negatively charged residues could be important for optimizing the interactions of peptides in the *m*AZ-pTyr-Xaa-Asn-NH_2 series with the Grb2 SH2 domain. Such a requirement could be more critical in the case of the (α-Me)pTyr "residue" as indicated by the loss of affinity previously reported in the monocharged carboxylate series.[22]

Finally, the phosphonate-containing peptides **P5**, **P6**, and **P7**, which bear two doubly negatively charged phosphates at physiological pH, show higher affinities for Grb2 than the monocharged carboxylate or phosphonomethyl-containing peptides. This enhancement is noteworthy in the case of peptide **P6**. Such a peptide, in which the phosphonate PO_3H_2 group is directly bound to the phenyl ring, is particularly interesting because it has an affinity close to that of phosphate peptide **P1** and should be more active in vivo than P1 because of its insensitivity to phosphatase.

Cellular Activity of the Peptide Prodrugs. Our approach to designing SH2 inhibitory agents encountered similar difficulties as in the design of antiviral or anticancer nucleoside analogues. Indeed, to become biologically active, nucleosides have to enter the cell and be converted into nucleotides by viral or cellular kinases; nucleotides might enter cells after coupling with enzymatically labile hydrophobic protecting groups. The kinases involved in nucleoside activation are substrate-specific, which limits the design of structural analogues thereof. Similar obstacles are encountered in the case of SH2 domain inhibitors. It could be possible to create pseudo-peptides with a phosphotyrosine residue and high affinity for the domain. Nevertheless, these molecules, even if they could enter the cell, would not be converted to the tyrosine phosphorylated analogue owing to the high specificity of cellular tyrosine kinases. Therefore, it is necessary to develop prodrugs of tyrosine phosphorylated molecules. Since lipophilicity is well-known as a prime physicochemical descriptor of drugs with relevance to their biological properties, the phosphate moiety can be masked by esterification to give compounds with increased lipophilicity. This prodrug approach is very useful for circumventing the kinase specificity because the inhibitor is already phosphorylated. Moreover, in the case of NSAIDs, such as those in the ibuprofen family, prodrugs exhibited improved therapeutic index.[52] Along these lines, McGuigan et al. have demonstrated delivery of masked phosphates of antiviral nucleosides inside living cells by resorting to phosphoramidate derivatives of amino acids, particularly in the case of alanine.[53] Such phosphoramidate protection was later applied by Gay et al. in the SH2–Grb2 inhibitor family.[17] Lipophilic α-acyloxyalkyl ester derivatives of phenyl phosphates were also described[54] and used in the structure of the phosphonopeptide inhibitor of the Src SH2 domain.[16] We have applied a strategy similar to that developed by the group of Imbach in the case of the antiviral drug AZT,[55,56] consisting of the introduction of *S*-acyl thioester groups on the phosphate moiety. These prodrugs enter cells and are degraded by esterases, following a slow multistep process. We have previously designed and synthesized Shc-derived (Ac-PFpYVNVP-NH_2) phosphopeptide SATE ester prodrugs. We resorted to an EGF-stimulated ER22 cellular model and showed such prodrugs to be able to enter cells and inhibit the Grb2–Shc interaction and Erk1 and Erk2 activation by MAP kinases. MeSATE prodrugs were also able to inhibit colony formation of NIH3T3/HER2 transformed cell lines on soft agar.[18] *t*BuSATE prodrugs, on the other hand, did not elicit these effects. The very high-affinity peptide **P1** was not able to diffuse into cells and was thus derivatized into double and mono di-MeSATE ester forms (**P10** and **P11**). In a similar cellular assay as described in ref 18, both prodrugs showed inhibition of the NIH3T3/HER2 cell growth on soft agar gel with IC_{50} values of 0.1–0.2 μM. The results are shown in Figure 2. Such IC_{50} values are very promising in terms of in vivo potential antitumor activity of these compounds. In preliminary experiments, we have shown that di-MeSATE ester **P10** inhibits 50% of MAP kinase activation induced by EGF in ER22 cells overexpressing the EGF receptor (data not shown) after 18 h of treatment, which suggests that **P10** might be cleaved, liberating phosphoinhibitors. Nevertheless, fluorescence transfer experiments between Grb2 and Shc or HER2 in NIH3T3 cells might be much more informative in terms of the peptide target.

The last double monobenzyl prodrug **P12** showed similar cellular activity, with IC_{50} ranging from 0.2 to 0.3 μM. Such results are in agreement with those of Chen et al., who had shown that MeSATE and benzyl are both prodrug protectors of the phosphinate group, the former releasing the inhibitor more quickly,[43] as well as with those of Joachim et al. who described that the benzyl ester of adenosine cyclic 3′,5′-phosphate was able to penetrate cells and to release high cellular levels of cAMP.[57]

Moreover, we have recently obtained results showing that a vectorized peptidimer that inhibits both SH3 domains of Grb2 tested on a nude mice model was able to block the growth of xenografted human tumor expressing Erb2/HER2 (Dr. M. F. Poupon, Institut

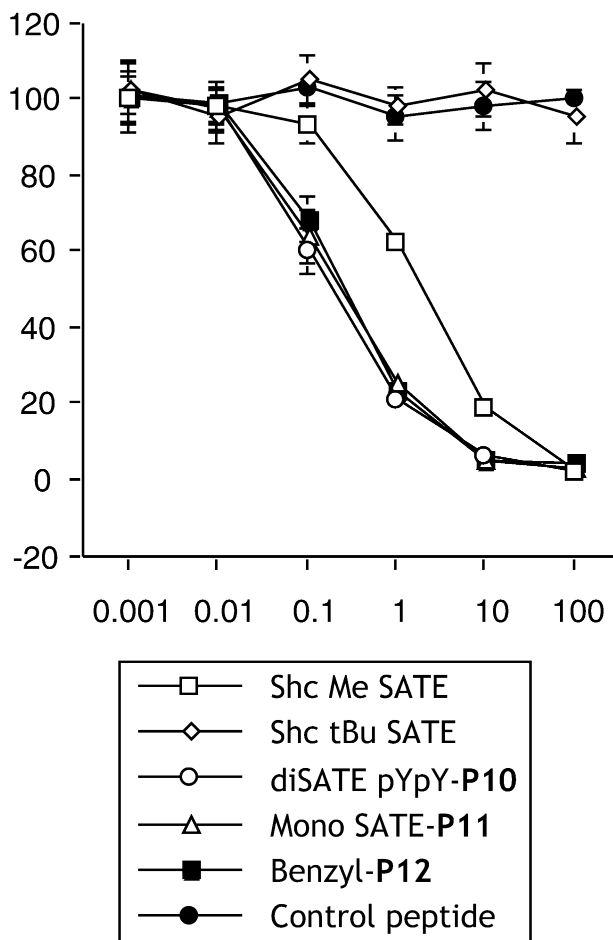


Figure 2. Inhibitory effect of SH2 inhibitors on colony formation of NIH3T3 cells transfected by HER2. Cells were grown on soft agar medium in the presence of different concentrations of inhibitors. Only colonies of about 100 cells or more were counted. The results are expressed as the percentage of colony as a function of inhibitor concentration (medium percentage of triplicate experiments ± SD). Typically, for 25 000 cells plated for the controls, 200 colonies were formed. The Shc sequence is Ac-PFpYVNVP-NH_2.[18] *t*BuSATE and MeSATE refer to *S*-pivaloyl and *S*-acetyl thioethylester.

Curie, France, unpublished results). From the same cloning efficiency test on soft agar, this peptidimer exhibited an IC_{50} value around 1 μM.[58] The 5- to 10-fold higher activity of the present phosphopeptide prodrugs could be anticipated to give rise to chemotherapeutic potential. Since IC_{50} values obtained here are very promising in terms of in vivo potential antitumor activity for these compounds, we are now exploring the pharmacological effect of these molecules on animal models.

Conclusion

We have shown in this paper that compounds that can efficiently disrupt Grb2−SH2 receptor interactions and hence inhibit Ras activation are able to block the growth of malignant cells that are dependent on the activation of growth factor receptor. We have developed phosphonomimetics of the α-methylphosphotyrosine and prodrugs active on cellular models especially as SATE or the monobenzyl ester of phosphopeptide. Antitumor tests on mice of prodrugs of the most active peptide **P1**, further developments of cell-permeable derivatives of peptide **P6**, and the search for nonpeptidic molecules are presently underway.

Experimental Section

Rink MBHA amide, Siber resins, and Fmoc-Tyr(PO_3Bzl)-OH were purchased from NovaBiochem. TFFH, HATU, and HOAt were from Perspective Biosystems. Fmoc-Tyr(PO_3-$MDPSE_2$)-OH was from Bachem Inc. The other reagents for solid-phase peptide synthesis were from Applied Biosystems, and the reagents for chemical preparations were from Aldrich.

The NMR spectra were recorded on a Bruker WH270 spectrometer operating at 270 MHz or at 400 MHz in the case of peptides. Chemical shifts are given in ppm relative to HMDS as the internal standard. The mass spectra were realized by the electrospray technique. C_{18} columns (Vydac, 5 μm) were used for analytical (4.6 mm × 150 mm) or preparative HPLC (10 mm × 250 mm). The UV detection was taken at 220 nm. The flows of the HPLC phases were 1 mL/min for analysis and 2 mL/min for semipreparation. The mobile phases used were the following: solvent A, H_2O with 0.1% TFA; solvent B, 70% CH_3CN with 0.09% TFA. The synthesis of peptides were realized on a 431A synthesizer of Applied Biosystem, programmed for Fmoc chemistry in small scale.

Affinity Measurement. Fluorescence measurements were performed on a LS250B Perkin-Elmer fluorimeter in a 10 mm × 10 mm cuvette at 25 °C, as described by Cussac et al.[58] The excitation was at 292 nm (bandwidth of 5.0 nm), and emission was recorded at 345 nm (bandwidth of 5.0 nm). The buffer was Hepes (50 mM, pH 7.5) and DTT (1 mM). The K_d constants were determined by the Michaelis−Menten type curve-fitting equation.

Competition Assay. Precoated streptavidin plates (Boehringer) were incubated with 100 μL/well of biotin-Ahx-PSpYVNVQN peptide (100 nM in PBS buffer) overnight at 4 °C. Nonspecific binding was blocked with PBS/3% BSA for 4 h at 4 °C. Competitors were incubated at the appropriate concentrations in PBS/3% milk containing 40 nM GST-Grb2 protein (100 μL/well) overnight at 4 °C. Revelation is made after anti-GST (Transduction Laboratories; 1/500 in PBS/milk/0.05% Tween 20) and peroxidase-coupled antimouse (Amersham; 1/1000 in PBS/milk/0.05% Tween 20) incubations, using TMB solution (Interchim). After coloration was stopped with H_2SO_4 (10% v/v), the optical density (OD) was read at 550 nm. Dose−reponse relationships were constructed by nonlinear regression of the competition curves with Origin 40 software.

Cell Culture. ER 22 cells were grown and lysed as described by Vidal et al.[59] NIH3T3 cells transfected with HER2 (a kind gift from Dr. A. Ullrich, Germany) were typically maintained in RPMI medium supplemented with 10% fetal calf serum (all from GIBCO).

Transformation Assays. The efficiency of colony formation in soft agar was determined by plating 25 000 NIH3T3/HER2 cells in 3 mL of 0.2% agar (GIBCO-BRL) in the presence of different concentrations of prodrug phosphopeptides. As described by Hudziak et al.,[60] increased expression of the putative growth factor receptor $p185^{HER2}$ causes transformation and tumorigenesis of NIH3T3 cells. After 2−4 weeks, colonies of about 100 cells or more were counted.

General Procedure for the Boc or Fmoc Protection of (α-Me)Tyr (Method A). To the suspension or the solution of (α-Me)Tyr or (α-Me)Tyr-OMe in dioxane/10% $NaHCO_3$ (1/1 in volume) was added in portions the di-*tert*-butyl dicarbonate (Boc_2O) or Fmoc-Cl (1.5−2.5 equivalents). The pH of the reaction mixture was maintained at 8−9 by occasional additions of 1 N NaOH. The reaction mixture was stirred at room temperature for 2−3 days before it was acidified to pH 2 by 1 N $KHSO_4$. The mixture was then extracted with EtOAc, and the organic extract was washed with water and brine and dried over Na_2SO_4. The residue of evaporation was purified by column chromatography on silica gel.

General Procedure of Tyrosine Phosphorylation (Method B). To a solution of Boc- or Fmoc-(α-Me)Tyr-OH in anhydrous THF was added *N*-methylmorpholine (1.1 equiv) followed by *tert*-butyldimethylsilyl chloride (1.2 equiv). The

solution was stirred at room temperature for 1 h. The solutions of 1*H*-tetrazole (4 equiv) and the phosphoramidite (4 equiv) in dry THF were introduced sucessively. The resulting mixture was stirred at room temperature for 3 h before cooling to 0 °C. The *tert*-butyl hydroperoxide (5−6 M in hexane, 5−6 equiv) was added, and the mixture was stirred for 30 min at 0 °C followed by another 30 min at room temperature. A 1 N $KHSO_4$ sample was added, and the mixture was extracted with EtOAc. The organic extract was washed with water and brine and dried over Na_2SO_4. After removal of the solvent, the residue was purified by column chromatography on silica gel to afford the product.

Fmoc-L-(α-Me)pTyr(MDPSE$_2$)-OH. Eluent: CH_2Cl_2/MeOH/AcOH (100/1/1). Yield: 80%. ^{1}H NMR (DMSO-d_6): δ 0.50 (s, 6H, 2 × MeSi), 1.1 (s, 3H, α-Me), 1.55 (m, 4H, 2 × CH_2Si), 2.85 and 3.2 (dd, 2H, $CH_2β$), 4.1 (q, 4H, 2 × CH_2O), 4.25 (m, 2H, 9-H and 9′-CH_2 of Fmoc), 4.5 (m, 1H, 9′-CH_2 of Fmoc), 6.88 (q, 4H, H−Ar of Tyr), 7.20 (s, 1H, NH), 7.32−7.85 (m, 28H, H−Ar of Ph and of Fmoc).

General Procedure for the Preparation of Compounds 3a−c (Method C). The para-substituted benzyl bromide (1.2 equiv) in anhydrous CH_2Cl_2 was added to a suspension of methyl (*N*-benzylidene)alaninate (1 equiv), KOH (1.5 equiv), K_2CO_3 (3 equiv), and benzyltrimethylammonium chloride (0.1 equiv) in dry CH_2Cl_2. The suspension was stirred at room temperature overnight, and the solids were removed by filtration. The solvent was evaporated, and the residue was dissolved in THF/5% citric acid (1/1 by volume). The resulting mixture was stirred at room temperature for 2 h. The organic solvent was removed by evaporation, and the aqueous residue was washed by ether to remove benzaldehyde. The aqueous solution was then neutralized by 10% $NaHCO_3$ to pH 9 and extracted with EtOAc. The crude product of the extraction workup was purified by column chromatography.

(D,L)-(α-Me)Pmp(*t*Bu$_2$)-OMe. Eluent: CH_2Cl_2/MeOH (95/5). Yield: 57%. ^{1}H NMR (DMSO-d_6 + TFA): δ 1.25 (s, 18H, 2 × *t*Bu), 1.40 (s, 3H, α-Me), 2.95 (d, 2H, CH_2−P), 3.0 (s, 2H, $CH_2β$), 3.65 (s, 3H, MeO), 7.0 and 7.15 (dd, 4H, H−Ar), 8.45 (s, 3H, NH_3^+).

(D,L)-(α-Me)F$_2$Pmp(Et$_2$)-OMe. Eluent: CH_2Cl_2/MeOH (95/5). Yield: 62%. ^{1}H NMR (DMSO-d_6): δ 1.15 (t, 9H, α-Me and 2 × CH_3 of Et), 1.85 (s, 2H, NH_2), 2.82 (q, 2H, $CH_2β$), 3.55 (s, 3H, MeO), 4.02 (q, 4H, 2 × CH_2 of Et), 7.22 and 7.40 (dd, 4H, H−Ar).

(D,L)-(α-Me)Phe(4-PO$_3$Et$_2$)-OMe or (D,L)-(α-Me)Ppp(Et$_2$)-OMe. Eluent: CH_2Cl_2/MeOH (95/5). Yield: 66%. ^{1}H NMR (DMSO-d_6): δ 1.15 (t, 9H, α-Me and 2 × CH_3 of Et), 1.80 (s, 2H, NH_2), 2.85 (q, 2H, $CH_2β$), 3.55 (s, 3H, MeO), 3.95 (q, 4H, 2 × CH_2 of Et), 7.25−7.55 (m, 4H, H−Ar).

General Procedure for the Preparation of Compounds 5, 5b, and 5c (Method D). To the solution of compound **3** in dioxane was added 1 N NaOH (1.2 equiv), and the resulting mixture was stirred at room temperature for 2 h before being neutralized to pH 9 by bubbling CO_2 into the mixture. The Fmoc-Cl (2 equiv) in dioxane was added, and the reaction mixture was maintained and worked up as described for method A.

Fmoc-(D,L)-(α-Me)Pmp(*t*Bu$_2$)-OH. The compound was purified by washing it through a pad of silica gel with CH_2Cl_2/MeOH, 98/2. Yield: 47%. ^{1}H NMR (DMSO-d_6 + TFA): δ 1.25 (s, 21H, 2 × *t*Bu and α-Me), 2.85 (d, 2H, CH_2−P), 3.05 (s, 2H, $CH_2β$), 4.2 (m, 3H, 9′-H and 9′-CH_2 of Fmoc), 6.95 (m, 4H, H−Ar of Pmp), 7.2−7.85 (m, 9H, H−Ar of Fmoc and NH).

Fmoc-(D,L)-(α-Me)F$_2$Pmp(EtH)-OH. Eluent: ($CHCl_3$/MeOH/H_2O/AcOH) (7/3/0.6/0.3)/EtOAc, 1/1. Yield: 41%. ^{1}H NMR (DMSO-d_6): δ 1.1 (m, 6H, α-Me and CH_3 of Et), 2.42 and 3.25 (dd, 2H, $CH_2β$), 3.95 (q, 2H, CH_2 of Et), 4.25 (m, 2H, 9′-H and 9′-CH_2 of Fmoc), 4.50 (m, 1H, 9′-CH_2 of Fmoc), 7.0−7.8 (m, 13H, H−Ar and NH).

Fmoc-(D,L)-(α-Me)Phe(4-PO$_3$Et$_2$)-OH. Eluent: CH_2Cl_2/MeOH (95/5). Yield: 23%.

^{1}H NMR (DMSO-d_6): δ 1.20 (t, 6H, 2 × CH_3 of Et), 1.35 (s, 3H, α-Me), 3.15 and 3.30 (dd, 2H, $CH_2β$), 3.95 (m, 4H, 2 × CH_2 of Et), 4.2 (m, 2H, 9′-CH_2 of Fmoc), 4.45 (m, 1H, 9′-H of Fmoc), 6.65 (s, 1H, NH), 7.1−7.8 (m, 12H, H−Ar).

General Procedure for the Preparation of Compounds 6 and 7 (Method E). Compound **5b** or **5c** was suspended in CH_3CN at room temperature. The TMSI was added, and the mixture was stirred at room temperature for 3 h before evaporation to dryness. The residue was then hydrolyzed in a cold mixture of TFA/H_2O/CH_3CN (1/1/2) for 30 min and then evaporated to dryness. The product was purified by column chromatography.

Fmoc-(D,L)-(α-Me)F$_2$Pmp-OH. Eluent: ($CHCl_3$/MeOH/H_2O/AcOH) (7/3/0.6/0.3)/EtOAc, 1/1. Yield: 86%. ^{1}H NMR (DMSO-d_6 + TFA): δ 1.12 (s, 3H, α-Me), 2.88 and 3.22 (dd, 2H, $CH_2β$), 4.22 (m, 2H, 9′-H and 9′-CH_2 of Fmoc), 4.48 (m, 1H, 9′-CH_2 of Fmoc), 7.0−7.85 (m, 13H, H−Ar and NH).

Fmoc-(D,L)-(α-Me)Phe(4-PO$_3$H$_2$)-OH. Eluent: ($CHCl_3$/MeOH/H_2O/AcOH) (7/3/0.6/0.3)/EtOAc, 2/1. Yield: 80%. ^{1}H NMR (DMSO-d_6 + TFA): δ 1.15 (s, 3H, α-Me), 2.9 and 3.25 (dd, 2H, $CH_2β$), 4.25 (m, 2H, 9′-CH_2 of Fmoc), 4.4 (m, 1H, 9′-H of Fmoc), 7.0−7.85 (m, 13H, H−Ar and NH).

General Procedure for the Synthesis of Peptides P1−P9 (Method F). These peptides were synthesized following the same method that was described for the synthesis of the peptide **P1**.[22] The suitably protected or free mimetics of pTyr were coupled successively to the Asn residue fixed on the Rink MBHA amide resin by the HATU/HOAt/DIEA (in ratio of 1/1/3 by equivalent to the amino acid). The N-terminal groups were introduced by coupling to Boc-*m*AZ-ONp. The peptides were then cleaved from the resin and deprotected by TFA/TIPS/H_2O (9.5/0.25/0.25) and purified by semipreparative HPLC.

***m*AZ-pTyr-(D,L)(α-Me)Pmp-Asn-NH$_2$ (P4).** MS, *m*/*z*: 801.2 for 778.6 (M + Na$^+$).

t_R = 8 min (0−80% of solvent B in 30 min, purity 96%). ^{1}H NMR (DMSO-d_6 + TFA): δ 1.1 and 1.25 (ss, 3H, α-Me), 2.5−3.2 (m, 8H, 3 × $CH_2β$ and CH_2−P), 4.2 (m, 1H, CHα), 4.35 (m, 1H, CHα), 4.9−5.05 (m, 2H, CH_2 of *m*AZ), 6.9−7.4 (m, 12H, H−Ar), 7.2 (t, 1H, NH), 7.25 and 8.0 (dd, 1H, NH), 8.35 and 8.55 (ss, 1H, NH of (α-Me)Pmp).

***m*AZ-pTyr-(D,L)(α-Me)F$_2$Pmp-Asn-NH$_2$ (P5).** MS, *m*/*z*: 815.4 for 815.2 (MH$^+$).

t_R = 13.5 min (5−35% of solvent B in 30 min, purity 96%). ^{1}H NMR (DMSO-d_6 + TFA): δ 1.1 and 1.25 (ss, 3H, α-Me), 2.5−3.35 (m, 6H, 3 × $CH_2β$), 4.25 (m, 2H, 2 × CHα), 4.9−5.0 (m, 2H, CH_2 of *m*AZ), 7.0−7.4 (m, 12H, H−Ar), 7.7 and 7.75 (dd, 1H, NH), 7.9 and 8.02 (dd, 1H, NH), 8.39 and 8.6 (ss, 1H, NH).

***m*AZ-pTyr-(L)(α-Me)Phe(4-PO$_3$H$_2$)-Asn-NH$_2$ (P6) or *m*AZ-pTyr-(D)(α-Me)Phe(4-PO$_3$H$_2$)-Asn-NH$_2$ (P7).** MS, *m*/*z*: 787.2 for 764.5 (M + Na$^+$).

P6. t_R = 11.0 min (5−35% of solvent B in 30 min, purity 97%). ^{1}H NMR (DMSO-d_6 + TFA): δ 1.25 (s, 3H, α-Me), 2.5−3.2 (m, 6H, 3 × $CH_2β$), 4.18 (m, 1H, CHα), 4.28 (m, 1H, CHα), 4.95 (q, 2H, CH_2 of *m*AZ), 7.05−7.5 (m, 12H, H−Ar), 7.7 (d, 1H, NH), 7.9 (d, 1H, NH), 8.3 (s, 1H, NH).

P7. t_R = 10.0 min (5−35% of solvent B in 30 min, purity 96%). ^{1}H NMR (DMSO-d_6 +TFA): δ 1.10 (s, 3H, α-Me), 2.6−3.35 (m, 6H, 3 × $CH_2β$), 4.25 (m, 1H, CHα), 4.35 (m, 1H, CHα), 5.0 (q, 2H, CH_2 of *m*AZ), 7.05−7.5 (m, 12H, H−Ar), 7.7 (d, 1H, NH), 8.1 (d, 1H, NH), 8.55 (s, 1H, NH).

***m*AZ-Pmp-(α-Me)pTyr-Asn-NH$_2$ (P8).** MS, *m*/*z*: 779.3 for 779.2 calculated, MH$^+$. t_R = 14.3 min (5−65% of solvent B in 30 min, purity 98%). ^{1}H NMR (DMSO-d_6 + TFA): δ 1.20 (s, 3H, α-Me), 2.5−2.8 (m, 4H, 2 × $CH_2β$), 2.9 (d, 2H, CH_2P), 3.05 (q, 2H, $CH_2β$), 4.25 (m, 2H, 2 × CHα), 4.95 (q, 2H, CH_2 of *m*AZ), 6.95−7.4 (m, 12H, H−Ar), 7.7 (d, 1H, NH), 7.82 (d, 1H, NH), 8.32 (s, 1H, NH).

***m*AZ-Pmp-(D,L)(α-Me)Pmp-Asn-NH$_2$ (P9).** MS, *m*/*z*: 777.2 for 776.6 calculated, MH$^+$. t_R = 17.0 min (5−35% of solvent B in 30 min, purity 97%). ^{1}H NMR (DMSO-d_6 + TFA): δ 1.1 and 1.25 (ss, 3H, α-Me), 2.5−3.25 (m, 8H, 3 × $CH_2β$ and CH_2P), 4.25 (m, 1H, CHα), 4.35 (m, 1H, CHα), 4.95−5.05 (m, 2H, CH_2 of *m*AZ), 6.9−7.45 (m, 12H, H−Ar), 7.7 (t, 1H, NH), 7.75 and 7.9 (dd, 1H, NH), 8.35 and 8.55 (ss, 1H, NH).

Diethyl (4-Methyl)phenylphosphonate. Diethyl phosphite (8.3 mL, 64.3 mmol), triethylamine (8.9 mL, 64.3 mmol), and tetrakis(triphenylphosphine)palladium (3.38 g, 2.92 mmol) were dissolved under nitrogen in 100 mL of toluene, and the solution was cooled to 0 °C. The 4-bromotoluene (10 g, 58.5 mmol) was then added, and the reaction mixture was brought to reflux for 3 h. After the mixture was cooled to room temperature, 250 mL of diethyl ether was added and the unsoluble solids were filtered off. The filtrate was evaporated to dryness and the residue was purified by column chromatography on silica gel (eluant: AcOEt/*c*-hexane, 1/1) to give 10.7 g of product as a yellow oil (yield, 80%). ^{1}H NMR (DMSO-d_6): δ 1.20 (t, 6H, 2 × CH_3 of Et), 2.35 (s, 3H, CH_3−Ar), 3.95 (q, 4H, 2 × CH_2 of Et), 7.3−7.6 (m, 4H, H−Ar).

Diethyl (4-Bromomethyl)phenylphosphonate (2c). The diethyl (4-methyl)phenylphosphonate was brominated with *N*-bromosuccinimide with dibenzoyl peroxide as catalyst (yield, 88%).[46] ^{1}H NMR (DMSO-d_6): δ 1.20 (t, 6H, 2 × CH_3 of Et), 4.0 (m, 4H, 2 × CH_2 of Et), 4.70 (s, 2H, CH_2Br), 7.1 (m, 4H, H−Ar).

(L)(α-Me)Tyr-OMe. To the suspension of (L)(α-Me)Tyr-OH (1.00 g, 5.12 mmol) in 25 mL of methanol, cooled in ice bath, was added dropwise 3.7 mL of thionyl chloride (51.2 mmol). The solution was stirred at 0 °C for half an hour and then heated to reflux overnight. After evaporation to dryness, 1.62 g of transparent oil (quantitative yield) was obtained. ^{1}H NMR (DMSO-d_6): δ 1.45 (s, 3H, α-Me), 2.95 (s, 2H, $CH_2\beta$), 3.65 (s, 3H, OCH_3), 6.7 and 6.9 (dd, 4H, H−Ar), 8.55 (s, 3H, NH_3^+), 9.5 (s, 1H, OH).

Boc-(L)(α-Me)Tyr-OMe. The compound was prepared following method A. Eluent: CH_2Cl_2/MeOH (98/2). Yield: 90%. ^{1}H NMR (DMSO-d_6): δ 1.10 (s, 3H, α-Me), 1.32 (s, 9H, Boc), 2.75 and 3.0 (dd, 2H, $CH_2\beta$), 3.55 (s, 3H, OCH_3), 6.6 and 6.8 (dd, 4H, H−Ar), 6.95 (s, 1H, NH), 9.15 (s, 1H, OH).

Boc-(L)(α-Me)Tyr(Tf)-OMe. Boc-(L)(α-Me)Tyr-OMe (1.2 g, 3.88 mmol) and *N*-phenyltrifluoromethanesulfonimide (1.52 g, 4.26 mmol) were dissolved under nitrogen in 12 mL of CH_2Cl_2. The solution was cooled to 0 °C, and then triethylamine (0.59 mL, 4.26 mmol) in 5 mL of CH_2Cl_2 was added. The reaction mixture was kept at room temperature for 3 h before the addition of 90 mL of ethyl ether. The organic solution was then washed successively with water (25 mL), 1 N NaOH (25 mL), water (25 mL), and brine (25 mL) and dried over Na_2SO_4. The residue obtained after evaporation of solvent was purified by column chromatography on silica gel (eluting with CH_2Cl_2) to give 1.37 g of white powder (yield, 80%). ^{1}H NMR (DMSO-d_6): δ 1.10 (s, 3H, α-Me), 1.32 (s, 9H, Boc), 2.9 and 3.3 (dd, 2H, $CH_2\beta$), 3.55 (s, 3H, OCH_3), 7.1 (s, 1H, NH), 7.2 and 7.35 (dd, 4H, H−Ar).

Boc-(L)(α-Me)Phe(4-PO$_3$Et$_2$)-OMe. A solution of Boc-(L)-(α-Me)Tyr(Tf)-OMe (1.00 g, 2.27 mmol), Pd(PPh_3)$_4$ (0.1 g, 0.086 mmol), *N*-methylmorpholine (0.32 mL, 2.95 mmol), and diethyl phosphite (0.4 mL, 2.72 mmol) in 6 mL of acetonitrile was heated to reflux under nitrogen overnight. The reaction mixture was then added to 100 mL of AcOEt, and the solution was washed successively with 5% $KHSO_4$ (3 × 50 mL), water (50 mL), saturated $NaHCO_3$ solution (3 × 50 mL), water (50 mL), and brine (50 mL) and dried over Na_2SO_4. The residue obtained after solvent evaporation was purified by column chromatography on silica gel (eluted with AcOEt/*c*-hexane, 6/4) to give 0.75 g of white powder (yield, 77%). ^{1}H NMR (DMSO-d_6): δ 1.10 (s, 3H, α-Me), 1.20 (t, 6H, 2 × CH_3 of Et), 1.32 (s, 9H, Boc), 2.9 and 3.3 (dd, 2H, $CH_2\beta$), 3.55 (s, 3H, OCH_3), 3.95 (q, 4H, 2 × CH_2 of Et), 7.05 (s, 1H, NH), 7.6 (q, 4H, H−Ar).

HCl (L)(α-Me)Phe(4-PO$_3$H$_2$)-OH. Boc-(L)(α-Me)Phe(4-PO$_3$Et$_2$)-OMe (0.7 g, 1.63 mmol) was refluxed in 9 N HCl (30 mL) overnight. The solution was then evaporated to dryness, and the residue was triturated with ethyl ether. The precipitate was collected by centrifugation, redissolved in water, and lyophilized to give 0.53 g of white powder (quantitative yield). ^{1}H NMR (DMSO-d_6): δ 1.45 (s, 3H, α-Me), 3.1 (s, 2H, $CH_2\beta$), 7.25 and 7.55 (mm, 4H, H−Ar), 8.45 (s, 3H, NH_3^+).

Fmoc-(L)(α-Me)Phe(4-PO$_3$H$_2$)-OH. This product was obtained following method A. ^{1}H NMR (DMSO-d_6 + TFA): δ 1.15 (s, 3H, α-Me), 2.95 and 3.25 (dd, 2H, $CH_2\beta$), 4.15 (t, 1H, 9′-H of Fmoc), 4.25 and 4.40 (mm, 2H, 9′-CH_2 of Fmoc), 7.20 (s, 1H, NH), 7.0 and 7.5 (mm, 4H, H−Ar of Phe), 7.3 (m, 4H, 2′, 3′, 6′, 7′-H of Fmoc), 7.65 (d, 2H, 4′, 5′-H of Fmoc), 7.8 (d, 2H, 1′, 8′-H of Fmoc).

Fmoc-(α-Me)pTyr(MeSATE$_2$)-OH. This compound was prepared according to the method described by Mathé et al.[50] Yield: 71%. ^{1}H NMR (DMSO-d_6): δ 1.12 (s, 3H, α-Me), 2.32 (s, 6H, 2 × CH_3CO), 2.85 and 3.15 (dd, 2H, $CH_2\beta$), 3.12 (t, 4H, 2 × CH_2S), 4.2 (m, 6H, 2 × CH_2O and 9′-CH_2 of Fmoc), 4.5 (m, 1H, 9′-H of Fmoc), 7.0 (q, 4H, H−Ar of Tyr), 7.25−7.9 (m, 9H, H−Ar of Fmoc and NH).

3-[*N*-(Fluorenylmethoxycarbonyl)amino]benzyl Alcohol. 3-Aminobenzyl alcohol (1.00 g, 8.1 mmol) was dissolved in 12.5 mL of THF and 8.1 mL of 1 N NaOH (8.1 mmol) cooled at 0 °C. To this solution was added Fmoc-Cl (3.15 g, 12.2 mmol), and the mixture was stirred at room temperature overnight. The organic solvent was then evaporated, and the aqueous residue that was acidified to pH 2 by 1 M $KHSO_4$ was extracted with AcOEt. The combined organic phase was washed successively with 1 M $KHSO_4$, H_2O, and brine and dried over Na_2SO_4. After filtration and solvent evaporation, the residue was purified by column chromatography on silica gel (eluent, 2% MeOH in CH_2Cl_2) to give 2.08 g product as white powder (yield, 74%). ^{1}H NMR (DMSO-d_6): δ 4.25 (q, 1H, 9′-H of Fmoc), 4.4 (m, 4H, 9′-CH_2 of Fmoc, CH_2O), 5.15 (t, 1H, OH), 6.9−7.9 (m, 12H, Ar−H), 9.7 (s, 1H, NH).

3-[*N*-(Fluorenylmethoxycarbonyl)amino]benzyl 4-Nitrophenylcarbonate (Fmoc-*m*AZ-ONp). 3-[*N*-(Fluorenylmethoxycarbonyl)amino]benzyl alcohol (1.85 g, 2.9 mmol) was dissolved in 12 mL of pyridine cooled at 0 °C. To this solution was added the 4-nitrophenyl chloroformiate (1.16 g, 5.75 mmol), and the mixture was stirred at room temperature overnight. The solvent was then evaporated, and the residue was redissolved in Et_2O, washed with H_2O and brine, and then dried over Na_2SO_4. After filtration and solvent evaporation, the residue was purified by column chromatography on silica gel (eluent, CH_2Cl_2) to give 366 mg of product as a white foam (yield, 14%). ^{1}H NMR (DMSO-d_6): δ 4.25 (t, 1H, 9′-H of Fmoc), 4.45 (d, 2H, 9′-CH_2 of Fmoc), 5.2 (s, 2H, CH_2O), 7.0−8.3 (m, 16H, H−Ar), 9.80 (s, 1H, NH).

***m*AZ-pTyr(MeSATE)$_2$-(α-Me)pTyr(MeSATE)$_2$-Asn-NH$_2$ (P10).** The prodrug was synthesized similarly to the other peptides but starting from Siber amide resin, which is more labile to acids. The side chain of Asn was protected by MeTrt, also more acid-labile than the usual Trt protection. Although it can be coupled in protection free form, in our studies of another phosphopeptide prodrugs, we have found that unprotected side chain results in 20% of peptide product dehydrated.[18] After classic coupling/deprotection of the residue Asn, the following two residues in Fmoc/MeSATE protections were coupled by the TFFH, and the Fmoc group was removed by 2% DBU in CH_2Cl_2. The last residue *m*AZ under active ester form was then introduced, and the N-terminal Fmoc was removed by 2% DBU. The resin cleavage and MeTrt deprotection were realized by addition of a solution of 25% TFA, 5% TIPS in CH_2Cl_2 (TFA/TIPS/CH_2Cl_2, 6 mL/0.6 mL/9 mL) at 0 °C for 30 min followed by another 30 min at room temperature. The resin was then filtered off, and the solution was cooled at 0 °C and neutralized to pH 8 by DIEA/CH_2Cl_2 (1/1 by volume). The mixture was then diluted with 30 mL of CH_2Cl_2 and washed with H_2O (2 × 30 mL) and brine and dried over Na_2SO_4. After solvent evaporation, the residue was purified by semipreparative HPLC on a Vydac C_{18} column: t_R = 16.0 min (40−65% of solvent B in 25 min, purity 97%). MS, *m*/*z*: 1189.16 for 1189.25 calculated, MH^+. ^{1}H NMR (DMSO-d_6): δ 1.22 (s, 3H, α-Me), 2.32 (s, 12H, 4 × CH_3CO), 2.4−3.0 (m, 6H, 3 × $CH_2\beta$), 3.1 (m, 8H, 4 × CH_2S), 4.1 (m, 8H, 4 × CH_2O), 4.2 (m, 2H, 2 × CHα), 4.78 (s, 2H, CH_2 of *m*AZ), 6.48 (bs, $CONH_2$), 6.55 (bs, $CONH_2$), 6.9−7.3 (m, 14H, H−Ar and NH_2 of *m*AZ), 7.58 (d, 1H, NH of Asn), 7.85 (d, 1H, NH of pTyr), 8.30 (s, 1H, NH of (α-Me)pTyr).

Boc-(L)(α-Me)Tyr-OH. Eluent: CH_2Cl_2/MeOH/AcOH (100/5/1). Yield: 60%. ^{1}H NMR (DMSO-d_6) : δ 1.1 (s, 3H, αMe), 1.3

(s, 9H, Boc), 2.75, 3.05 (dd, 2H, $CH_2\beta$), 6.6, 6.85 (dd, 4H, H−Ar), 6.5 (s, 1H, NH), 9.15, (s, 1H, OH).

Boc-(α-Me)Tyr-Asn-NH$_2$. Boc-(α-Me)Tyr-OH (2.5 g, 8.46 mmol) and HCl·Asn-NH$_2$ (1.70 g, 10.15 mmol) were dissolved in 60 mL of DMF. The solution was adjusted to pH 9 by the DIEA (about 5 mL). The HOAt (1.61 g, 11.85 mmol) and the HATU (4.5 g, 11.85 mmol) were then added, and the mixture was stirred at room temperature for 3 days before evaporation to dryness. The residue was purified by chromatography with CH_2Cl_2/MeOH/H_2O/AcOH (7/3/0.6/0.3)/AcOEt, 1:1, as eluent. An amount of 2.96 g of white solid was obtained with a yield of 86%. ^{1}H NMR (DMSO-d_6): δ 1.1 (s, 3H, αMe), 1.35 (s, 9H, Boc), 2.3 (m, 2H, $CH_2\beta$Asn), 2.75, 2.90 (dd, 2H, $CH_2\beta$Tyr), 4.5 (m, 1H, ChaAsn), 6.55, 6.85 (dd, 4H, H−Ar), 6.8−7.1 (m, 4H, 2CONH$_2$), 7.3 (s, 1H, NHTyr), 8.0 (d, 1H, NHAsn), 9.15, (s, 1H, OH).

Boc-(α-Me)pTyr(MeSATE)$_2$-Asn-NH$_2$. Boc-(αMe)Tyr-Asn-NH$_2$ (1.20 g, 2.93 mmol) was phosphorylated with bis(*S*-acetyl-2-thioethyl)*N*,*N*-diéthylphosphoramidite as described.[18] The crude product was purified by chromatography with CH_2Cl_2/MeOH/AcOH (100/5/1) as eluent. An amount of 2.34 g of white solid was obtained with 69% yield. ^{1}H NMR (DMSO-d_6): δ 1.15 (s, 3H, αCH_3), 1.41 (s, 9H, Boc), 2.3 (s, 6H, 2 × CH_3CO), 2.4−2.6 (m, 2H, $CH_2\beta$Asn), 2.85, 3.1 (dd, 2H, $CH_2\beta$Tyr), 3.15 (t, 4H, 2 × CH_2S), 4.15 (q, 4H, 2 × CH_2O), 4.3 (m, 1H, CHαAsn), 6.8, 7.1 (ss, 4H, 2 × CONH$_2$), 7.1 (q, 4H, H−Ar Tyr), 7.25 (s, 1H, NH−Tyr), 8 (d, 1H, NH Asn).

Boc-(α-Me)pTyr(Bzl$_2$)-Asn-NH$_2$. Boc-(αMe)Tyr-Asn-NH$_2$ (0.5 g, 1.22 mmol) was phosphorylated with dibenzyl *N*,*N*-diethylphosphoramidite as described.[51] The crude product was purified by chromatography with CH_2Cl_2/MeOH/AcOH (100/5/1) as eluent. An amount of 0.49 g of white solid was obtained with 60% yield. ^{1}H NMR (DMSO-d_6): δ 1.15 (s, 3H, αCH_3), 1.40 (s, 9H, Boc), 2.3−2.6 (m, 2H, $CH_2\beta$Asn), 2.93, 3.1 (dd, 2H, $CH_2\beta$Tyr), 4.25 (m, 1H, CHαAsn), 5.1 (d, 4H, CH_2 Bzl), 7.05 (q, 4H, H−Ar Tyr), 7.25 (m, 11H, H−Ar Bzl and NH Tyr), 7.3 (m, 4H, 2 × CONH$_2$), 8 (d, 1H, NH Asn).

(α-Me)pTyr(MeSATE)$_2$-Asn-NH$_2$. A cold solution of TFA/CH_2Cl_2 (1/1, 15 mL) was added to Boc-(α-Me)pTyr(MeSATE)$_2$-Asn-NH$_2$ (2.24 g, 3.23 mmol). The mixture was stirred at 0 °C for 1 h. Heptane (50 mL) was added, and the solution was evaporated to dryness. The residue was washed with ether to give a white solid (2.48 g, quantitative yield). ^{1}H NMR (DMSO-d_6): δ 1.15 (s, 3H, αCH_3), 2.25 (s, 6H, 2 × CH_3CO), 2.35−2.6 (m, 2H, $CH_2\beta$Asn), 2.95, 3.2 (dd, 2H, $CH_2\beta$Tyr), 3.15 (t, 4H, 2 × CH_2S), 4.1 (q, 4H, 2 × CH_2O), 4.6 (m, 1H, CHαAsn), 7.1 (d, 4H, H−Ar Tyr), 7.3 (m, 4H, 2 × CONH$_2$), 7.9 (s, 3H, NH_3^+-Tyr), 8.5 (d, 1H, NH Asn).

(α-Me)pTyr-Asn-NH$_2$. This product was obtained by treating Boc-(α-Me)pTyr(Bzl$_2$)-Asn-NH$_2$ (0.48 g, 0.72 mmol) with TFA/CH_2Cl_2 (1/1) for 3 h at room temperature. The residue obtained from evaporation was washed with ether and collected by centrifugation (0.34 g, yield, 93%). ^{1}H NMR (DMSO-d_6): δ 1.4(s, 3H, αCH_3), 2.4 (m, 2H, $CH_2\beta$Asn), 2.85, 3.1 (dd, 2H, $CH_2\beta$Tyr), 4.5 (m, 1H, CHαAsn), 7.05, 7.2 (dd, 4H, H−Ar Tyr), 7.3 (m, 4H, 2 × CONH$_2$), 8.0 (s, 3H, NH_3^+Tyr), 8.6 (d, 1H, NH Asn).

Boc-pTyr(MeSATE$_2$)-(α-Me)pTyr(MeSATE)$_2$-Asn-NH$_2$. Boc-pTyr(MeSATE)$_2$-OH (2.11 g, 3.73 mmol) and (α-Me)-pTyr(MeSATE)$_2$-Asn-NH$_2$ (2.40 g, 3.39 mmol) were coupled with 1.1 equiv of HATU/HOAT as described for the preparation of Boc-(α-Me)Tyr-Asn-NH$_2$. The crude product after evaporation was taken up in AcOEt, washed with 10% NaHCO$_3$, 10% citric acid, and brine and dried over Na$_2$SO$_4$. The residue obtained after evaporation of solvent was purified by chromatography with CH_2Cl_2/MeOH/AcOH (100/5/1) as eluent to give 2.55 g of product (yield 60%). ^{1}H NMR (DMSO-d_6): δ 1.2 (s, 12H, αMe + Boc), 2.3 (s, 12H, 4 × CH_3CO), 2.5−3 (m, 14H, 3 × $CH_2\beta$ + 4 × CH_2S), 4.1 (m, 10H, 2CHα + 4 × CH_2O), 6.85 (m, 4H, 2 × CONH$_2$), 7.05 (m, 8H, H−Ar), 7.3 (d, 2H, NH Tyr + NH αMe Tyr), 7.9 (d, 1H, NH Asn).

Boc-pTyr(MeSATE)$_2$-(α-Me)pTyr-Asn-NH$_2$. Boc-pTyr-(MeSATE)$_2$-OH (0.48 g, 0.85 mmol) and (α-Me)pTyr-Asn-NH$_2$ (0.33 g, 0.85 mmol) were coupled with 1.1 equiv of HATU/HOAT as described for the preparation of Boc-(α-Me)Tyr-Asn-NH$_2$. The crude product after evaporation of solvent was purified by the semipreparative HPLC to give 29 mg of product (yield, 3.6%). ^{1}H NMR (DMSO-d_6): δ 1.25 (s, 12H, αMe + Boc), 2.3 (s, 6H, 4 × CH_3CO), 2.5−3 (m, 6H, 3 × $CH_2\beta$), 3.1 (t, 4H + 2 × CH_2S), 4.05−4.2 (m, 6H, 2CHα + 2 × CH_2O), 6.85−7.3 (m, 13H, 8 × H−Ar + NH Tyr + 2 × CONH$_2$), 7.8 (d, 1H, NH Asn), 8.25 (s, 1H, + NH αMeTyr).

pTyr(MeSATE$_2$)-(α-Me)pTyr(MeSATE)$_2$-Asn-NH$_2$. This product was obtained by the deprotection of Boc as described for the preparation of (α-Me)pTyr(MeSATE)$_2$-Asn-NH$_2$ with 85% yield. ^{1}H NMR (DMSO-d_6): δ 1.25 (s, 3H, αMe), 2.25 (s, 12H, 4 × CH_3CO), 2.3−2.6 (m, 14H, 3 × $CH_2\beta$ + 4 × CH_2S), 3.9−4.15 (m, 8H, 4 × CH_2O), 4.2 (m, 2H, CHα), 7 (m, 8H, H−Ar Tyr), 7.15 (d, 4H, 2 × CONH$_2$), 7.3 (s, 1H, NH αMe Tyr), 8 (s, 3H, NH_3^+ αMeTyr), 8.6 (d, 1H, NH Asn).

Boc-*m*AZ-pTyr(MeSATE)$_2$-(α-Me)pTyr(MeSATE)$_2$-Asn-NH$_2$. H_3N^+-pTyr(MeSATE)$_2$-(αMe)pTyr(MeSATE)$_2$-Asn-NH$_2$ (1.85 g, 1.61 mmol) in DMF was adjusted to pH 9 by DIEA addition. Boc-*m*AZ-ONp (0.69 g, 1.77 mmol) was added, and the mixture was stirred at room temperature for 5 days. The solvent was then evaporated and the residue was purified by chromatography with CH_2Cl_2/MeOH/AcOH (100/5/1) as eluent to give 1.88 g of product (yield, 91%). ^{1}H NMR (DMSO-d_6): δ 1.2 (s, 3H, αMe), 1.4 (s, 9H, Boc), 2.3 (s, 12H, 4 × CH_3CO), 2.5−3 (m, 6H, 3 × $CH_2\beta$), 3.1 (m, 8H, 4 × CH_2S), 4−4.3 (m, 10H, 2CHα + 4 × CH_2O), 4.8 (q, 2H, $CH_2\phi$), 6.85 (m, 4H, 2 × CONH$_2$), 7−7.5 (m, 12H, H−Ar), 7.7 (d, 1H, NH Tyr), 7.9 (d, 1H, NH Asn), 8.3 (s, 1H, NH αMe Tyr), 9.3 (s, 1H, NH *m*AZ).

***m*AZ-pTyr(MeSATE)$_2$-(α-Me)pTyr(MeSATE)$_2$-Asn-NH$_2$ (P10).** The product was obtained by the deprotection of Boc-*m*AZ-pTyr(MeSATE)$_2$-(α-Me)pTyr(MeSATE)$_2$-Asn-NH$_2$ (1.87 g, 1.45 mmol) as described for the preparation of (α-Me)pTyr-(MeSATE$_2$)-Asn-NH$_2$. The final product was purified by semipreparative HPLC to give 1.15 g of product (yield, 61%). It has the same characteristics as those observed in the previous synthesis (purity in HPLC, 98%).

***m*AZ-pTyr(MeSATE)$_2$-(α-Me)pTyr-Asn-NH$_2$ (P11).** The deprotection of Boc-pTyr(MeSATE)$_2$-(α-Me)pTyr-Asn-NH$_2$ was performed as described for the preparation of (α-Me)pTyr-(MeSATE)$_2$-Asn-NH$_2$. The product obtained (22 mg, 0.023 mmol) was coupled with Boc-*m*AZ-ONp (18 mg, 0.046 mmol). The crude product Boc-*m*AZ-pTyr(MeSATE)$_2$-(α-Me)pTyr-Asn-NH$_2$, obtained after evaporation of the solvent, was submitted to deprotection of the Boc group. The final product was purified by semipreparative HPLC to give 6 mg of final product (yield, 26%). MS, *m*/*z*: 985.0 for 984.23 calculated. t_R = 11.0 min (0−80% of solvent B in 30 min, purity 98%).

Fmoc-Asp(*t*Bu)-NH-(CH$_2$)$_3$-(1-naphthyl). Fmoc-Asp(*t*Bu)-OH (1.11 g, 2.7 mmol) and 3-(1-naphthyl)-1-propylamine[21] (0.50 g, 2.7 mmol) were dissolved in 10 mL of DMF. BOP (1.2 g, 2.7 mmol) and then DIEA (0.95 mL, 5.4 mmol) were added, and the solution was stirred at room temperature overnight. DMF was then evaporated, and the residue was taken in ethyl acetate. The solution was washed with 1 M KHSO$_4$, saturated NaHCO$_3$, water, and brine before drying with anhydrous Na$_2$SO$_4$. After filtration, the filtrate was evaporated to dryness and the residue was purified by chromatography with EtOAc/*c*-hexane as eluent to give 1.3 g of white powder (yield, 83%). ^{1}H NMR (CDCl$_3$): δ 1.3 (s, 9H, *t*Bu), 1.85 (m, 2H, 2-CH_2 of propyl), 2.55 and 2.85 (dd, 2H, $CH_2\beta$ of Asp), 3.0 (t, 2H, CH_2 of naphthyl), 3.3 (m, 2H, CH_2−N), 4.15 (t, 1H, CHα of Asp), 4.4 (m, 3H, 9-H and CH_2 of Fmoc), 5.85 (bs, 1H, NH-propyl), 6.45 (bs, 1H, NH of Asp), 7.2−8.0 (m, 15H, H−Ar of Fmoc and naphthyl).

Fmoc-Asp-NH-(CH$_2$)$_3$-(1-naphthyl). Fmoc-Asp(*t*Bu)-NH-(CH$_2$)$_3$-(1-naphthyl) (1.3 g, 2.25 mmol) was dissolved in 4 mL of 50% TFA in CH_2Cl_2 with 1% anisol. The solution was stirred for 15 min at 0 °C after 4 h at room temperature. The solvent was then evaporated, and the residue was precipitated with a solution of petroleum ether/diethyl ether (2/1). The precipitate was collected by centrifugation and washed three times with a mixture of petroleum ether/diethyl ether to give 1.1 g of white powder (yield, 94%). ^{1}H NMR (DMSO-d_6): δ 1.70 (m, 2H,

2-CH_2 of propyl), 2.45 and 2.70 (qq, 2H, $CH_2\beta$ of Asp), 2.95 (t, 2H, CH_2−naphthyl), 3.15 (m, 2H, CH_2−N), 4.2 (m, 4H, CHα of Asp, 9-H and CH_2 of Fmoc), 7.25−8.0 (m, 17H, 2 NH and H−Ar of Fmoc and naphthyl).

Fmoc-(α-Me)pTyr (Bzl)-OH. Fmoc-(α-Me)pTyr$(Bzl)_2$-OH, obtained by following ref 51 (600 mg, 0.885 mmol), was dissolved in 3 mL of acetonitrile. NaI (132 mg, 1.77 mmol) was added, and the suspension was refluxed for 2 h. After evaporation of the solvent, the residue was taken in water and washed thoroughly with diethyl ether until the aqueous phase became colorless.[51] Lyophilization of the aqueous phase gave 410 mg of a white powder (yield, 88%). ^{1}H NMR (DMSO-d_6): δ 1.20 (s, 3H, α-CH_3), 2.85 and 3.10 (dd, 2H, $CH2\beta$), 4.2 (m, 2H, CH_2 of Fmoc), 4.4 (m, 1H, 9-H of Fmoc), 4.70 (d, 2H, CH_2 of Bzl), 6.75 and 6.90 (dd, 4H, H−Ar of pTyr), 7.2−7.85 (m, 9H, NH and H−Ar of Fmoc).

Ac-pTyr(Bzl)-(α-Me)pTyr(Bzl)-Asn-NH−$(CH_2)_3$-naphthyl (P12). The Siber resin (300 mg, 0.1 mmol) was deprotected by a solution of 2% DBU in DMF and washed thoroughly with DMF. A mixture of Fmoc-Asp-NH-$(CH_2)_3$-(1-naphthyl) (1 mmol), HATU (1 mmol), HOAt (1 mmol), and DIEA (3 mmol) in 3.5 mL of DMF was added to the deprotected Siber resin, and the coupling was maintained for 4 h before resin draining and washing. The Fmoc group deprotection and the HATU/HOAt/DIEA coupling were repeated to introduce protected (α-Me)pTyr and pTyr residues. After removal of the final Fmoc protection, the peptidyl resin was capped in a solution of acetic anhydride (5 mmol)/DIEA (5 mmol) in DMF (3 mL). After being washed with DMF and then CH_2Cl_2, the peptidyl resin was dried in a vacuum and cleaved with a solution of 2% TFA in CH_2Cl_2 (5 mL) for 5 min. The cleavage was repeated five times. The cleavage filtrates were combined and coevaporated several times with cyclohexane to give the crude product, which was purified by semipreparative HPLC on a C_{18} Vydac column. Lyophilization of the product fractions gave 32 mg of **P12** as a white powder. MS, *m/z*: 1088.3 for 1022, M + $3Na^+$. t_R = 24.5 min (10−90% of solvent B in 30 min, purity 95%).

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