Synthesis and Structure of Cyclic Phosphopeptides Containing a Phosphodiester Linkage

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The synthesis of three cyclic phosphopeptides, which contain a phosphodiester linkage, is described. Starting from either Boc-L-Ser(OBn)-OH or Boc-L-Thr(OBn)-OH, three precursors for the macrocyclization by phosphitylation were prepared. After phosphitylation, using 4-chlorobenzyl bis(*N*,*N*-diisopropylamino)phosphinite or 4-chlorobenzyl dichlorophosphinite, subsequent oxidation and hydrogenolysis the cyclic phosphopeptides **18**–**20** were obtained. The solution conformation of cyclic phosphopeptide **18** was studied by NMR spectroscopy and restrained molecular dynamics calculations.

Introduction

In 1988, Live and Edmondson[1] demonstrated by two-dimensional ^{1}H–^{31}P NMR the presence of an earlier proposed[2] phosphodiester linkage between the hydroxyl groups of serine and threonine in *Azotobacter vinelandii* flavodoxin. Additional evidence for the presence of a phosphodiester linkage between two amino acid residues in proteins came from ^{31}P NMR studies on glucose oxidase from *Aspergillus niger*[3] and 6-hydroxyl-L-nicotine oxidase from *Arthrobacter oxidans*.[4]

This led to the proposal[2,3,5,6] that a phosphodiester linkage could stabilize or retain the protein secondary structure in a manner similar to that of disulfide bridges in proteins.[7-9] In particular, the phosphodiester linkage might be able to stabilize a turn. This view is also supported by comparison of the properties of the native *Azotobacter* phosphoprotein with those of the recombinant dephosphoprotein by Taylor *et al.*[6]

As part of a program on the synthesis and structure of phospho amino acids and phosphopeptides,[5,10] we have described the synthesis of a cyclic molecule containing a phosphodiester linkage between a serine and threonine residue, which could serve as a model for an *intramolecular* phosphodiester cross-link in a protein.[5] The results of Taylor *et al.*[6] favor both *intramolecular*[11] linkages (*viz.* Ser[129]-Thr[130] and Ser[157]-Thr[160])[12] rather than the *intermolecular* linkage.

Since macromolecules containing a phosphate moiety are not widely studied,[13] it was a challenge to synthesize cyclic peptides containing a phosphodiester linkage.[5] The structure of the cyclic peptides is affected by the presence of this *intramolecular* linkage, which might be capable of stabilizing a turn. The presence of a P=O moiety could also lead to interesting receptor molecules, which might be able to bind metal ions and organic cations.[13,14,21]

In this paper, we wish to report an integral study describing the synthesis of three cyclic phosphopeptides and the structural analysis—by NMR—of one of its congeners. These cyclic phosphopeptides can be considered as model compounds for peptides containing *intramolecular* phosphodiester linkages. Both the synthesis and structure were a challenge because of the presence of the charged phosphate moiety in a ring. This might have a considerable influence on the conformation due to solvation, presence of a counterion, and (in the solid-state) on the packing.

Results and Discussion

A. Synthesis. The synthesis of the precursors for the macrocyclization is straightforward and outlined in Scheme I. Starting from commercially available Boc-Ser(OBn)-OH (**1**) and Boc-Thr(OBn)-OH (**2**), the corresponding amides **3** and **4** were prepared[15] with yields of 80% and 86%, respectively. Subsequent removal of the Boc group, followed by coupling of Boc-Gly-OH by the mixed anhydride method,[16] gave dipeptides **5** and **6** in 82% and 90%

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(1) Live, D. H.; Edmondson, D. E. *J. Am. Chem. Soc.* **1988**, *110*, 4468.

(2) (a) Edmondson, D. E.; James, T. L. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 3786. (b) Edmondson, D. E.; James, T. L. In *Flavine & Flavoproteins*; Massey, V., Williams, C. H., Eds.; Elsevier North Holland: Amsterdam, 1982; p 11.

(3) James, T. L.; Edmondson, D. E.; Husain, M. *Biochemistry* **1981**, *20*, 617.

(4) Pust, S.; Vervoort, J.; Decker, K.; Bacher, A.; Müller, F. *Biochemistry* **1989**, *28*, 516.

(5) Van Oijen, A. H.; Erkelens, C.; Van Boom, J. H.; Liskamp, R. M. J. *J. Am. Chem. Soc.* **1989**, *111*, 9103.

(6) Taylor, M. F.; Boylan, M. H.; Edmondson, D. E. *Biochemistry* **1990**, *29*, 6911.

(7) Stryer, L. In *Biochemistry*, 2nd ed.; W.N. Freeman and Company: New York, 1981.

(8) Srinivasan, N.; Sowdhamini, R.; Ramakrishnan, C.; Balaram, P. *Int. J. Pept. Protein Res.* **1990**, *36*, 147.

(9) Light, A. *Proteins, Structure and Function*; Prentice-Hall Inc.: Englewood Cliffs, NJ, 1975.

(10) (a) De Bont, H. B. A.; Veeneman, G. H.; Van Boom, J. H.; Liskamp, R. M. *J. Recl. Trav. Chim. Pays-Bas* **1987**, *106*, 641. (b) De Bont, H. B. A.; Liskamp, R. M. J.; O'Brian, C. A.; Erkelens, C.; Veeneman, G. H.; Van Boom, J. H. *Int. J. Pept. Protein Res.* **1989**, *33*, 115. (c) De Bont, H. B. A.; Van Boom, J. H.; Liskamp, R. M. J. *Tetrahedron Lett.* **1990**, *31*, 2497. (d) De Bont, D. B. A.; Moree, W. J.; Van Boom, J. H.; Liskamp, R. M. J. *J. Org. Chem.* **1993**, *58*, 1309.

(11) The term "*intramolecular*" is used here to indicate a phosphodiester linkage between two hydroxy amino acids with at the most two amino acid residues in between, whereas the term "*intermolecular*" refers to a phosphodiester linkage between two hydroxy amino acids which are further apart.

(12) Drummond, M. H. *Eur. J. Biochem.* **1986**, *159*, 549.

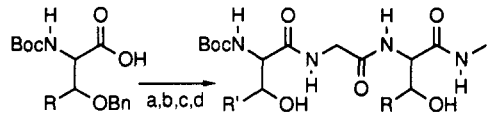
(13) For a recent review on phosphorus-containing macrocycles: Tsvetkov, E. N.; Bovin, A. N.; Syundyukova, V. Kh. *Usp. Khim.* **1988**, *57*, 1353.

(14) The P=O moiety in, e.g., triphenylphosphine is a good proton acceptor and can form complexes with a variety of organic bases; see, e.g.; Etter, M. C.; Baures, P. W. *J. Am. Chem. Soc.* **1988**, *110*, 639 and references cited therein.

(15) Perich, J. W.; Johns, R. B. *J. Org. Chem.* **1988**, *53*, 4103.

0022-3263/93/1958-3722$04.00/0

Scheme I[a]



[a] Key: (a) (i) isobutyl chloroformate, 4-methylmorpholine, (ii) H_2NMe; **3**, R = H (80%); **4**, R = CH_3 (86%); (b) (i) TFA/CH_2Cl_2, (ii) Boc-Gly-OH, isobutyl chloroformate, 4-methylmorpholine, Et_3N; **5**, R = H (82%); **6**, R = CH_3 (90%); (c) (i) TFA/CH_2Cl_2, (ii) DCC, HOBt, Boc-Thr(OBn)-OH or Boc-Ser(OBn)-OH, 4-methylmorpholine; **7**, R = H, R' = CH_3 (89%); **8**, R = R' = H (84%); **9**, R = R' = CH_3 (83%); (d) 10% Pd/C, H_2, MeOH; **10**, R = H, R' = CH_3 (98%); **11**, R = R' = H (95%); **12**, R = R' = CH_3 (94%).

yield, respectively. The protected tripeptides **7–9** were obtained in good yields (89%, 84%, and 83%, respectively) after coupling of Boc-Thr(OBn)-OH or Boc-Ser(OBn)-OH by the DCC/HOBt method.[17] Hydrogenolysis of the benzyl protecting groups led to the macrocyclization precursors Boc-Thr(OH)-Gly-Ser(OH)-$NHCH_3$ (**10**, 98%), Boc-Ser(OH)-Gly-Ser(OH)-$NHCH_3$ (**11**, 95%), and Boc-Thr(OH)-Gly-Thr(OH)-$NHCH_3$ (**12**, 94%).

Phosphorylation of the tripeptides **10–12** was accomplished using the phosphite triester method, as is shown in Scheme II. To a solution[18] containing tripeptide **10**, **11**, or **12** and 2 equiv of 1*H*-tetrazole was slowly added a solution of 4-chlorobenzyl bis(*N*,*N*-diisopropylamino)-phosphinite[19] (**13**). The intermediate phosphite triester was not isolated but directly oxidized using *tert*-butyl hydroperoxide. Subsequent purification by flash chromatography[20] afforded the cyclic phosphotriesters **15–17** (Table I, method A).

Since the cyclic phosphopeptides **15–17** were obtained in relatively moderate yields (30%, 15%, and 28%, respectively), we tried to improve the yield by using the more reactive phosphitylating agent **14**, which had been crucial in the synthesis of a phosphodiester linkage containing amino acid based cryptand.[21]

Indeed, addition of diisopropylethylamine and 4-chlorobenzyl dichlorophosphinite (**14**) to a solution containing tripeptide **10**, **11**, or **12** and subsequent oxidation with 3-chloroperbenzoic acid more than doubled the yield of the cyclic phosphotriesters, which were obtained in yields of 62%, 70% and 62%, respectively (Table I, method B).

Although the diastereomeric ratios of the triesters are not important in the synthesis of the cyclic phosphopeptides **18–20**, the dependency of the phosphitylating agent is interesting: using the more reactive phosphitylating agent for a faster phosphitylation gives rise to a decreased diastereomeric ratio (Table I).[22]

Using *tert*-butyl hydroperoxide instead of 3-chloroperbenzoic acid for the oxidation of the intermediate phosphite triester in method B did not affect the yield(s). At first, slow addition of the phosphitylating agent was thought necessary to avoid diphosphitylation (method A). However, experiments showed that diphosphitylation products could not be detected upon fast addition of the phosphitylating agent. Thus, the higher yields obtained using method B are the result of the more reactive 4-chlorobenzyl dichlorophosphinite and not due to the formation of less byproducts.

Hydrogenolysis of the 4-chlorobenzyl group under buffered[23] conditions, *i.e.*, in the presence of sodium acetate, afforded the sodium salt of the cyclic phosphopeptides **18–20** in, respectively, 95%, 92%, and 93% yield (Scheme II). Surprisingly, if hydrogenolysis under these conditions was followed by treatment with TFA, the Boc group could only be removed with difficulty or not at all from the N-terminus. For complete removal of the Boc group, it was necessary to carry out the hydrogenolysis in the absence of sodium acetate—during which the resulting phosphodiester acid already partly cleaved the Boc group—followed by treatment with TFA. So far, we have no insight as to the refractory nature of **18–20** toward TFA.

B. Structure. The development of a reliable conformation from NMR requires the diastereotopic assignment of all geminal protons. This was achieved, in the case of **18**, by use of homonuclear coupling constants and qualitative evaluation of the C^1–C^3H cross peaks from the HMBC spectrum following a procedure described in the literature.[24] These assignments were checked by NOE effects.[25]

From the vicinal coupling $^3J(NH,C^2H)$ for both Gly-C^2H (see experimental details), using the corresponding Karplus relation,[26] the only possible ϕ-backbone angles are ±73° and ±89°. An additional Karplus equation for the heteronuclear long-range coupling $^3J(C^2H(i),C^1(i\text{-}1))$, by which the ϕ angle is also accessible, and extraction of $C^2H(i)$–$C^1(i\text{-}1)$ cross peak intensities from a HMBC spectrum (Figure 1) leads unambiguously to $\phi = \pm 89°$. In this case, there is principally no way to distinguish the sign, but the result of the MD simulations justifies the assignment. In the same spectrum the weak serine C^1–C^3H_a and C^1–C^3H_b signals correspond to two small gauche couplings, suggesting the conformation to be staggered with $\chi_1 = 60°$. This could not be confirmed with the homonuclear $^3J(C^2H,C^3H)$ because of strong coupling, which leads to a higher order coupling pattern. However, the assignment of Ser-C^3H_a (*pro-S*) and Ser-C^3H_b (*pro-R*)

(16) Bodanszky, M.; Bodanszky, A. In *The Practice of Peptide Chemistry—Reactivity and Structure Concepts in Organic Chemistry*; Hafner, K., Rees, C. W., Trost, B. M., Lehn, J.-M., Schleyer, P. v. R., Zahradnik, R. Eds.; Springer-Verlag: New York, 1984; Vol. 21, p 109.

(17) Reference 16, p 145.

(18) The starting concentration of tripeptide **10–12** was 50 mM; more diluted solutions to avoid *intermolecular* reactions did not improve the yields. Surprisingly, dimeric products could not be detected in the reaction mixture, not even if the concentration was increased by a factor 2.

(19) Phosphitylating agent **13** was prepared from PCl_3 in two steps analogous to the procedure described for benzyl bis(*N*,*N*-diisopropylamino)phosphinite by Dreef *et al.* (Dreef, C. E.; Elie, C. J. J.; Hoogerhout, P.; Van der Marel, G. A.; Van Boom, J. H. *Tetrahedron Lett.* **1988**, *29*, 6513). In view of the lability of this phosphitylating agent, it is advisable to prepare compound **13** when needed. Due to the lability of the phosphitylating agent and the slow addition, it is necessary to use 2 equiv of the agent because of decomposition during the addition. This reagent was also prepared by Caruthers *et al.* (Caruthers, M. H.; Kierzek, R.; Tang, J. Y. In *Biophosphates and Their Analogues—Synthesis, Structure, Metabolism and Activity*; Bruzik, K. S., Stec, W. J., Eds.; Elsevier Science Publishers: Amsterdam, 1987; p 3) in a different manner.

(20) Still, W. C.; Kahn, M.; Mitra, A. *J. Org. Chem.* **1978**, *43*, 2923.

(21) Van Oijen, A. H.; De Bont, H. B. A.; Van Boom, J. H.; Liskamp, R. M. J. *Tetrahedron Lett.* **1991**, *32*, 7723.

(22) A satisfactory explanation for the observed diastereomeric ratios cannot be given. The presence of both a serine and a threonine residue and a slow phosphitylation seem necessary to give a (large) difference in stereoselectivity.

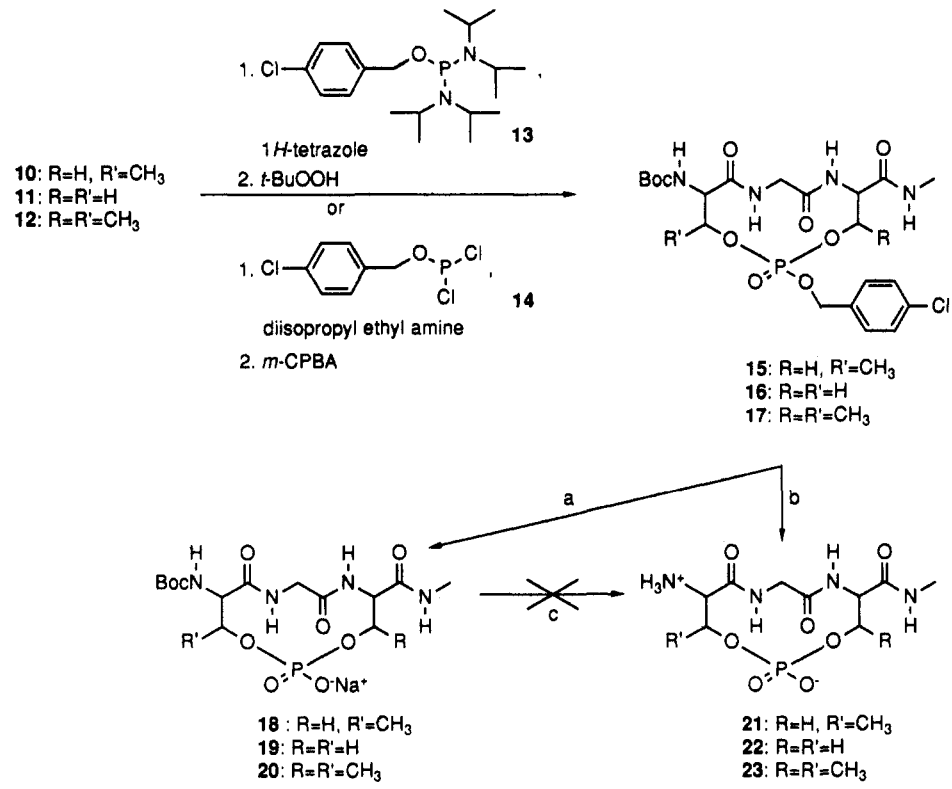
(23) De Bont, H. B. A.; Van Boom, J. H.; Liskamp, R. M. J. *Recl. Trav. Chim. Pays-Bas* **1990**, *109*, 27.

(24) (a) Hofmann, M.; Bermel, W.; Gehrke, M.; Kessler, H. *Magn. Reson. Chem.* **1989**, *27*, 877. (b) Bermel, W.; Wagner, K.; Griesinger, C. *J. Magn. Reson.* **1989**, *83*, 223.

(25) Wagner, G.; Braun, W.; Havel, T. F.; Schaumann, T.; Go, N.; Wüthrich, K. *J. Mol. Biol.* **1987**, *196*, 611.

(26) Bystrov, V. F. *Progr. NMR Spectr.* **1976**, *10*, 41.

Scheme II[a]



[a] Key: (a) 10% Pd/C, H_2, NaOAc, *t*-BuOH/H_2O; (b) (i) 10% Pd/C, H_2, *t*-BuOH/H_2O, (ii) TFA/CH_2Cl_2; (c) TFA/CH_2Cl_2.

Table I. Yields and Diastereomeric Ratio of the Cyclic Phosphopeptides 15–17

	method A		method B	
compd	yield (%)	ratio	yield (%)	ratio
15	30	15:1	62	4.2:1
16	15	1:1	70	2.2:1
17	28	3:1	62	1:1

is easily confirmed by the strong NOE between Ser-NH and Ser-C^3H_b.

To examine the diastereotopic assignment of the glycine C^2H protons, the method of floating chirality was utilized.[27] First the protons in question, *i.e.*, C^2H_a and C^2H_b, were assigned a prochirality, *pro-R* and *pro-S*, and the NOE restraints developed using this assignment. Then for the angles in which the carbon C^2 is in the middle (*i.e.*, C^2H–C^2–C^2H, N–C^2–C^2H, etc.) the force constant is set to zero. Therefore, the positions (prochirality) of the protons can switch during the MD simulation to meet the experimental measurements, *i.e.*, NOEs. If the assignment is wrong, the protons will switch their stereogenic positions. Here, since there was only one questionable center, the method was carried out for both of the possible assignments. In one, the protons switched positions, and in the other the prochirality assignments were maintained during the simulation.

The partially energy-minimized average structure from the simulation is shown in Figure 2. The 12-membered ring system adopts a conformation similar to a βII′ turn: the torsions about C^1–C^2–C^3–O and C^3–C^2–C^1–N (involving the side chain of threonine) and the ϕ and ψ of glycine are 37, −137, −63, and −16°, respectively (see Table II). This should be compared to the values for a standard βII′ turn,

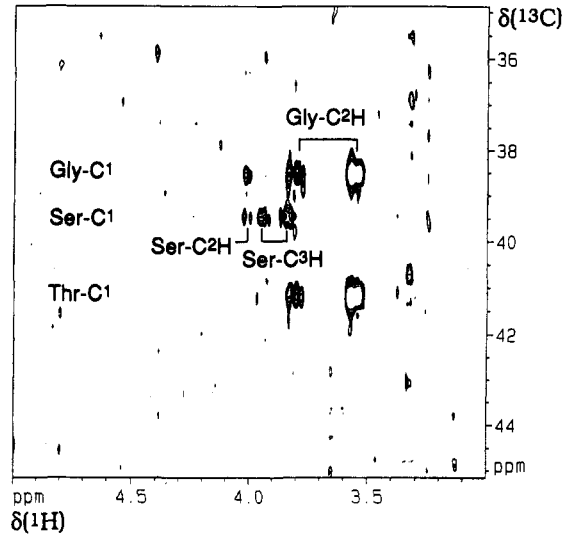


Figure 1. HMBC spectrum of 18. Carbonyl resonances are folded twice into a small spectral window of 65 ppm. The scale of the carbon axis is not correct for the carbonyl resonances because of the folding (twice) of the spectrum in this dimension. The Ser-C^1–C^3H_a/C^3H_b correlations are of nearly equal intensity, which corresponds to a small coupling constant between both C^3H protons and the carbonyl group. In the calculations C^1 is in anticlinal position to both C^3H protons (see text and Table IV).

60, −120, −80, and 0°, for the ϕ and ψ of the second and third residues.

The constraint of the cyclic system is apparent in the conformation of the side chain of serine. Surprisingly, an almost eclipsed rotamer is observed for the average structure (120° for the N–C^2–C^3–O dihedral angle). This is of course disfavored by the force field. However, the NOEs between the HN of the methylamide end group

(27) Holak, T. A.; Gondol, D.; Otlewski, J.; Wilusz, T. *J. Mol. Biol.* **1989**, *210*, 635.

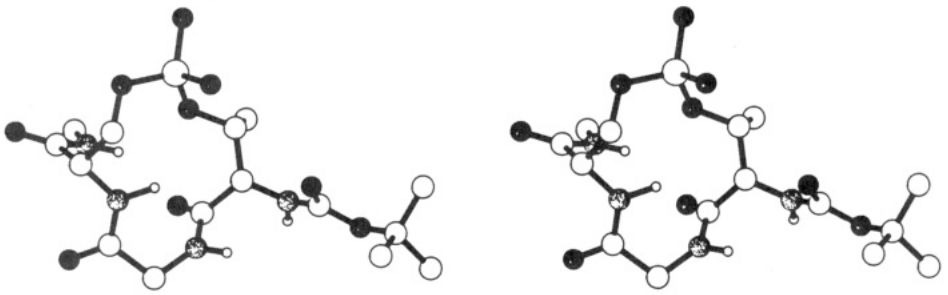


Figure 2. Stereoplot of the partially minimized (200 steps of steepest descent minimization) average structure from the 100-ps MD simulation of 18 in DMSO.

Table II. Average Dihedral Angles during MD Simulation of 18 in DMSO

residue	dihedral	av (deg)
Boc	$C(CH_3)OC^1N$	180
	ω	178
Thr	ϕ	−127
	ψ	76
	ω	165
	$NC^2C^3C^4$	65
	NC^2C^3O	−177
	C^2C^3OP	118
	C^3OPO	−150
	$C^1C^2C^3O$	37
	$C^3C^2C^1N$	−137
Gly	ϕ	−63
	ψ	−16
	ω	175
Ser	ϕ	−68
	ψ	−31
	ω	177
	NC^2C^3O	120
	$C^1C^2C^3C^3H_a$	114
	$C^1C^2C^3C^3H_b$	−152
	C^2C^3OP	−113
	C^3OPO	59

($NHCH_3$ in Table III) and the threonine forces the end group back over the cyclic portion of the molecule, resulting in the observed dihedral. It is important to note that the standard deviation of this torsion during the simulation is much larger than the others (35°) indicating the competing forces of the NOE restraints on the one hand and MD force field on the other. The analysis of the coupling constants, noted above, indicated the 60° rotamer for this side chain. This rotamer was determined from the equal intensity of the C^1–C^2–C^3–C^3H cross peaks in the HMBC. The two dihedral angles (included in Table II) giving rise to the HMBC peaks are indeed of equal magnitude (when considering the large standard deviation).

Conclusions

Cyclic phosphopeptides are accessible by the phosphite triester method. When 4-chlorobenzyl dichlorophosphinite was used as the phosphitylating agent the cyclic phosphopeptides containing a phosphodiester linkage were obtained in 62–70% yield. The yields were less satisfactory (15–30%) when the amidite method, *i.e.*, using 4-chlorobenzyl bis(*N*,*N*-diisopropylamino)phosphinite, was employed. It is expected that other cyclic phosphopeptides can be prepared using this method. The syntheses of cyclic phosphotetrapeptides of Ser-Gly-Lys-Thr, as may be present in *Azotobacter* flavodoxin, as well as Ser-Ala-Ala-Ser, which may form a stabilized secondary structure element, are presently under investigation.

Table III. Interproton Distances of 18 Determined by Integration of the Corresponding ROESY Crosspeaks, Compared to Values from Molecular Dynamics Simulations

proton 1	proton 2	distance (pm)	MD (pm)
Thr-NH	Thr-C^3H	368	334
Thr-NH	Thr-C^4H_3	298	310
Thr-NH	Gly-NH	264	250
Thr-NH	$C(CH_3)_3$	387	385[a]
Thr-NH	$NHCH_3$	492	505
Thr-C^2H	Thr-C^3H	240	202
Thr-C^2H	Thr-C^4H_3	255	313
Thr-C^2H	$C(CH_3)_3$	341	474[a]
Thr-C^3H	Thr-C^4H_3	232	212
Gly-NH	Gly-C^2H_a (*pro-S*)	266	260
Gly-NH	Gly-C^2H_b (*pro-R*)	293	269
Gly-NH	Thr-C^2H	314	240
Gly-NH	Thr-C^3H	360	394
Gly-NH	Ser-NH	268	269
Gly-NH	$NHCH_3$	474	420
Ser-NH	Ser-C^2H	287	289
Ser-NH	Ser-C^3H_b (*pro-R*)	283	251
Ser-NH	Thr-C^2H	361	257
Ser-NH	Thr-C^3H	351	373
Ser-NH	Thr-C^4H_3	395	423
Ser-NH	Gly-C^2H_a (*pro-S*)	328	310
Ser-NH	$NHCH_3$	304	220
Ser-NH	$NHCH_3$	430	422[b]
$NHCH_3$	$NHCH_3$	253	234[b]
$NHCH_3$	Thr-C^2H	291	382
$NHCH_3$	Thr-C^3H	516	425
$NHCH_3$	Thr-C^4H_3	460	395
$NHCH_3$	Gly-C^2H_a (*pro-S*)	399	402
$NHCH_3$	Ser-C^2H	303	299
$NHCH_3$	Ser-C^3H_b (*pro-R*)	326	342

[a] 200 pm was added to the upper distance restraint. [b] 30 pm was added to the upper distance restraint.

NMR spectroscopy in combination with restrained molecular dynamics simulations were used to study the structure of the cyclic phosphopeptide 18 in solution.

Experimental Section

A. Synthesis. General. Unless otherwise stated, chemicals were obtained from commercial sources and used without further purification. Isobutyl chloroformate (IBCF) was distilled under argon. "Dry" solvents were distilled immediately prior to use from an appropriate drying agent. Tetrahydrofuran (THF), ether, and dioxane were distilled from $LiAlH_4$. 4-Methylmorpholine, CH_2Cl_2, and CH_3CN were distilled from CaH_2. Reactions were monitored by thin-layer chromatography (TLC) on Merck precoated silica gel 60 F254 (0.25 mm) using CH_2Cl_2/MeOH, 9/1, v/v as eluent or on Merck precoated silica gel 60 F254, silanized (RP-2, 0.25 mm) using MeOH/H_2O, 1/1, v/v. Compounds were visualized by UV light and by dipping in a solution of ninhydrin followed by heating the plate for a few minutes. Short column chromatography was performed on silica gel 60 (Merck, 230–400 mesh ASTM), with the indicated eluents. Flash chromatography[20] was performed on silica gel 60H (Merck) using CH_2Cl_2/MeOH, 95/5, v/v, as eluent. Sephadex LH20 (Pharmacia) was

used for gel filtration. Organic layers, obtained after washing procedures, were dried on $MgSO_4$, filtered, and concentrated *in vacuo*.

Melting points are uncorrected. ^1H and ^{13}C NMR spectra were measured on a 200-MHz, a 300-MHz, or a 600-MHz spectrometer, operating in the Fourier transform mode. ^{31}P NMR spectra were measured on a 200-MHz apparatus. TMS was used as internal and 85% H_3PO_4 as external standard. ^{13}C NMR spectra were measured using the attached proton test (ATP)[28] technique. Spectra necessary for determination of the solution conformation were recorded at 300 K on a Bruker AMX 500 spectrometer equipped with a Bruker Aspect X32 computer for processing. The numbering of the carbon atoms in the amino acids is according to IUPAC recommendations.[29] The compounds were homogeneous according to NMR and TLC.

Boc-Ser(OBn)-NHCH$_3$ (3). This amide was prepared according to the procedure described by Perich and Johns[15] starting from Boc-Ser(OBn)-OH (1, 2.50 g, 8.47 mmol). After the reaction mixture was stirred for 3 h compound **3** was isolated by adding 1 N aqueous $KHSO_4$ (50 mL) to the reaction mixture and stirring for 10 min at rt. Ethyl acetate (EtOAc) (100 mL) was added, and the organic layer was washed with 1 N aqueous $KHSO_4$ (2 × 50 mL), 5% aqueous $NaHCO_3$ (2 × 50 mL), and brine (50 mL). Crystallization from EtOAc/hexane (1/3, v/v) gave **3**, isolated as white crystals (6.78 mmol, 2.09 g) in 80% yield: R_f = 0.53; mp = 95 °C; ^1H NMR ($CDCl_3$) δ 1.44 (s, 9 H, Boc, $C(CH_3)_3$), 2.82 (d, 3 H, $NHCH_3$, J = 6.0 Hz), 3.57 and 3.91 (eight lines, AB of ABX, 2 H, Ser-C^3H_2, J_{AX} = 6.2 Hz, J_{BX} = 3.9 Hz, J_{AB} = 9.3 Hz), 4.28 (m, 1 H, Ser-C^2H), 4.51 and 4.56 (2d, 2 H, benzyl, $PhCH_2$, J = 11.8 Hz), 5.42 (m, 1 H, Ser-NH), 6.47 (m, 1 H, $NHCH_3$), 7.32 (m, 5 H, benzyl, $PhCH_2$, aromatic part); ^{13}C NMR ($CDCl_3$) δ 26.0 ($NHCH_3$), 28.1 and 79.8 (Boc), 53.9 (Ser-C^2), 69.8 and 73.0 (Ser-C^3, benzyl, $PhCH_2$), 127.4, 127.6, 128.2 and 137.4 (benzyl, $PhCH_2$, aromatic part), 170.6 (Ser-C^1).

Boc-Thr(OBn)-NHCH$_3$ (4). The above-described procedure was used to prepare amide **4** starting from Boc-Thr(OBn)-OH (**2**, 3.10 g, 10.02 mmol). Crystallization from EtOAc/hexane gave **4**, isolated as white crystals (2.78 g, 8.62 mmol) in 86% yield: R_f = 0.81; mp = 126 °C; ^1H NMR ($CDCl_3$) δ 1.16 (d, 3 H, Thr-C^4H_3, J = 6.2 Hz), 1.45 (s, 9 H, Boc, $C(CH_3)_3$), 2.82 (d, 3 H, $NHCH_3$, J = 4.9 Hz), 4.19 (m, 2 H, Thr-C^2H, Thr-C^3H), 4.56 and 4.60 (2d, 2 H, benzyl, $PhCH_2$, J = 11.6 Hz), 5.50 (d, 1 H, Thr-NH), 6.48 (m, 1 H, $NHCH_3$), 7.32 (m, 5 H, benzyl, $PhCH_2$, aromatic part); ^{13}C NMR ($CDCl_3$) δ 15.6 (Thr-C^4), 26.2 ($NHCH_3$), 28.3 (Boc), 57.7 (Thr-C^2), 71.7 (benzyl, $PhCH_2$), 74.8 (Thr-C^3), 127.7, 127.8 and 128.4 (benzyl, $PhCH_2$, aromatic part).

Boc-Gly-Ser(OBn)-NHCH$_3$ (5). To a solution of Boc-Ser(OBn)-$NHCH_3$ (**3**, 1.01 g, 3.27 mmol) in dry CH_2Cl_2 (10 mL) at 0 °C was added trifluoroacetic acid (TFA) (10 mL), and the mixture was stirred for 1 h at 0 °C. The reaction mixture was evaporated and coevaporated with dry ether (5 × 5 mL). The residue was dissolved in dry THF (2 mL) and neutralized with Et_3N to an apparent pH of 7–8. To a solution of Boc-Gly-OH (585 mg, 3.34 mmol) in dry THF (10 mL) at −10 °C were added 4-methylmorpholine (0.40 mL, 3.64 mmol) and IBCF (0.45 mL, 3.47 mmol). After 10 min the solution of the above-prepared amine in THF was added. The reaction mixture was stirred for 2 h at −10 °C, and 1 N aqueous $KHSO_4$ (25 mL) was added. After the reaction mixture was stirred for 10 min at rt, EtOAc (50 mL) was added and the organic layer was washed with 1 N aqueous $KHSO_4$ (2 × 25 mL), 5% aqueous $NaHCO_3$ (2 × 25 mL), and brine (25 mL). Short column purification (20 g silica, eluent: CH_2Cl_2/MeOH 95/5, v/v) gave **5** (980 mg, 2.68 mmol) in 82% yield as a colorless solid: R_f = 0.28; mp = 117 °C; ^1H NMR ($CDCl_3$) δ 1.42 (s, 9 H, Boc, $C(CH_3)_3$), 2.76 (d, 3 H, $NHCH_3$, J = 4.9 Hz), 3.57 and 3.98 (8 lines, AB of ABX, 2 H, Ser-C^3H_2, J_{AX} = 5.4 Hz, J_{BX} = 3.6 Hz, J_{AB} = 9.3 Hz), 3.75 (d, 2 H, Gly-C^2H_2, J_{HNH} = 5.4 Hz), 4.42 and 4.53 (2d, 2 H, benzyl, $PhCH_2$, J = 11.8 Hz), 4.59 (m, 1 H, Ser-C^2H), 5.69 (t, 1 H, Gly-NH), 7.06 (m, 1 H, $NHCH_3$), 7.18 (d, 1 H, Ser-NH, J = 8.0 Hz), 7.33 (m, 5 H, benzyl, $PhCH_2$, aromatic part); ^{13}C NMR ($CDCl_3$) δ 26.2 ($NHCH_3$), 28.1 and 80.3 (Boc), 44.5 (Gly-C^2), 52.8 (Ser-C^2), 69.3 and 73.2 (Ser-C^3, benzyl, $PhCH_2$), 127.6, 127.7, 128.3 and 137.4 (benzyl, $PhCH_2$, aromatic part), 169.7 and 170.1 (Ser-C^1, Gly-C^1).

Boc-Gly-Thr(OBn)-NHCH$_3$ (6). The Boc-group of compound **4** (2.49 g, 7.73 mmol) was removed in CH_2Cl_2/TFA (1/1, v/v). The resulting TFA salt, Boc-Gly-OH (1.42 g, 8.12 mmol), 4-methylmorpholine (0.91 mL, 8.13 mmol), and IBCF (1.05 mL, 8.10 mmol) in dry THF were used to prepare **6** according to the procedure for the preparation of compound **5**. Purification on a silica gel column (150 g of silica, eluent: CH_2Cl_2/MeOH, 96/4, v/v) afforded **6** (2.64 g, 6.96 mmol) as a white solid in 90% yield: R_f = 0.31; mp = 139 °C; ^1H NMR ($CDCl_3$) δ 1.15 (d, 3 H, Thr-C^4H_3, J = 6.4 Hz), 1.39 (s, 9 H, Boc, $C(CH_3)_3$), 2.77 (d, 3 H, $NHCH_3$, J = 4.6 Hz), 3.75 (d, 2 H, Gly-C^2H_2, J_{HNH} = 5.9 Hz), 4.26 (m, 1 H, Thr-C^2H), 4.47 (m, 1 H, Thr-C^3H), 4.48 and 4.57 (2d, 2 H, benzyl, $PhCH_2$, J = 11.6 Hz), 5.77 (t, 1 H, Gly-NH), 7.20 (m, 7 H, Thr-NH, $NHCH_3$, benzyl, $PhCH_2$, aromatic part); ^{13}C NMR ($CDCl_3$) δ 16.1 (Thr-C^4), 26.1 ($NHCH_3$), 28.0, 80.2 and 156.5 (Boc), 44.5 (Gly-C^2), 56.9 (Thr-C^2), 71.4 (benzyl, $PhCH_2$), 74.0 (Thr-C^3), 127.4, 127.5, 128.2 and 137.9 (benzyl, $PhCH_2$, aromatic part), 169.9 and 170.1 (Gly-C^1, Thr-C^1).

Boc-Thr(OBn)-Gly-Ser(OBn)-NHCH$_3$ (7). The Boc group of **5** (903 mg, 2.47 mmol) was removed in CH_2Cl_2/TFA (1/1, v/v), according to the procedure described for the deprotection of compound **3**. To the resulting mixture were added Boc-Thr(OBn)-OH (817 mg, 2.64 mmol) and 1-hydroxybenzotriazole (HOBt) (338 mg, 2.50 mmol). The mixture was cooled to 0 °C, and after 10 min *N,N*-dicyclohexylcarbodiimide (DCC) (556 mg, 2.69 mmol) was added. After 15 min the pH was adjusted to pH 7–8 by addition of 4-methylmorpholine and the cooling was removed. Stirring was continued for 3 h at ambient temperature, and 1 N aqueous $KHSO_4$ (25 mL) and EtOAc (50 mL) were subsequently added. The organic layer was washed with 1 N aqueous $KHSO_4$ (2 × 25 mL), saturated aqueous $NaHCO_3$ (2 × 25 mL), and brine (25 mL). Purification on a silica gel column (35 g silica, eluent: CH_2Cl_2/MeOH, 98/2, v/v) gave **7** (1.23 g, 2.20 mmol) as a white solid in 89% yield: R_f = 0.53; mp = 126 °C; ^1H NMR ($CDCl_3$) δ 1.17 (d, 3 H, Thr-C^4H_3, J = 6.2 Hz), 1.42 (s, 9 H, Boc, $C(CH_3)_3$), 2.71 (d, 3 H, $NHCH_3$, J = 4.1 Hz), 3.55 and 3.78 (8 lines, AB of ABX, 2 H, Ser-C^3H_2, J_{AX} = 6.2 Hz, J_{BX} = 4.6 Hz, J_{AB} = 9.3 Hz), 3.96 (m, 2 H, Gly-C^2H_2), 4.05 (m, 1 H, Thr-C^2H), 4.44, 4.45, 4.51 and 4.52 (4d, 4 H, 2 benzyl, $PhCH_2$, J = 11.3, 11.8 Hz), 4.41 (m, 1 H, Ser-C^2H), 4.69 (m, 1 H, Thr-C^3H), 5.76 (d, 1 H, Thr-NH, J = 8.2 Hz), 7.33 (m, 13 H, Ser-NH, Gly-NH, $NHCH_3$, 2 benzyl, $PhCH_2$, aromatic part); ^{13}C NMR ($CDCl_3$) δ 15.8 (Thr-C^4), 26.0 ($NHCH_3$), 28.1, 79.7 and 155.8 (Boc), 43.1 (Gly-C^2), 52.8 (Ser-C^2), 58.2 (Thr-C^2), 69.4, 71.1 and 72.9 (Ser-C^3, 2 benzyl, $PhCH_2$), 74.6 (Thr-C^3), 127.4, 128.0, 128.1, 137.4 and 137.8 (2 benzyl, $PhCH_2$, aromatic part), 168.7, 170.0 and 171.1 (Ser-C^1, Gly-C^1, Thr-C^1).

Boc-Ser(OBn)-Gly-Ser(OBn)-NHCH$_3$ (8). The Boc group of **5** (1.28 g, 2.51 mmol) was removed in CH_2Cl_2/TFA (1/1, v/v) according to the procedure described for the deprotection of compound **3**. To the resulting solution were added Boc-Ser(OBn)-OH (1.01 g, 3.43 mmol), HOBt (490 mg, 3.62 mmol), and DCC (766 mg, 3.71 mmol) according to the procedure for the preparation of compound **7**. Purification on a silica gel column (40 g of silica, eluent: CH_2Cl_2/MeOH, 98/2, v/v) afforded **8** (1.59 g, 2.93 mmol) as a white solid in 84% yield: R_f = 0.63; mp = 131 °C; 300-MHz ^1H NMR ($CDCl_3$) δ 1.43 (s, 9 H, Boc, $C(CH_3)_3$), 2.76 (d, 3 H, $NHCH_3$, J = 4.8 Hz), 3.57 and 3.83 (8 lines, AB of ABX, 2 H, Ser-C^3H_2, J_{AX} = 6.6 Hz, J_{BX} = 4.6 Hz, J_{AB} = 9.5 Hz), 3.59 and 3.86 (8 lines, AB of ABX, 2 H, Ser'-C^3H_2, J_{AX} = 5.9 Hz, J_{BX} = 4.6 Hz, J_{AB} = 9.5 Hz), 3.90 and 3.95 (8 lines, AB of ABX, 2 H, Gly-C^2H_2, J_{AB} = 26.7 Hz, J_{ANH} = 5.2 Hz, J_{BNH} = 5.0 Hz), 4.32 (m, 1 H, Ser-C^2H), 4.48 and 4.52 (2d, 4 H, 2 benzyl, $PhCH_2$, J = 12.0 Hz), 4.58 (m, 1 H, Ser'-C^2H), 5.50 (d, 1 H, Ser-NH, J = 6.9 Hz), 6.77 (q, 1 H, $NHCH_3$), 7.11 (d, 1 H, Ser'-NH, J = 7.6 Hz), 7.31 (m, 11 H, Gly-NH, 2 benzyl, $PhCH_2$, aromatic part); ^{13}C NMR ($CDCl_3$) δ 26.0 ($NHCH_3$), 28.1, 79.8 and 155.5 (Boc), 43.2 (Gly-C^2), 52.8 and 54.3 (2 Ser-C^2), 69.5 and 69.9 (2 Ser-C^3), 72.9 (2 benzyl, $PhCH_2$), 127.4, 127.5, 128.1 and 137.4 (2 benzyl, $PhCH_2$, aromatic part), 168.7, 170.0 and 171.1 (2 Ser-C^1, Gly-C^1).

(28) (a) Brown, D. H.; Nakashima, T. F.; Rabenstein, D. R. *J. Magn. Reson.* **1981**, *45*, 303. (b) Ben, R.; Gunter, H. *Angew. Chem., Int. Ed. Engl.* **1983**, *22*, 350.

(29) IUPAC-IUB, Nomenclature and Symbolism for Amino Acids and Peptides. Recommendations 1983. *J. Biol. Chem.* **1984**, *260*, 14.

Boc-Thr(OBn)-Gly-Thr(OBn)-NHCH$_3$ (9). After removal of the Boc-group of compound **6** (1.52 g, 4.01 mmol) by TFA in CH_2Cl_2 (1/1, v/v), the TFA salt was dissolved in dry THF. Boc-Thr(OBn)-OH (1.36 g, 4.21 mmol), HOBt (572 mg, 4.23 mmol) and DCC (872 mg, 4.23 mmol) were added to prepare **9** according to the procedure described for the preparation of compound **7**. Compound **9** (1.89 g, 3.31 mmol) was isolated after purification on a silica gel column (60 g silica, eluent: CH_2Cl_2/MeOH, 95/5, v/v) as a white crystalline material in 83% yield: R_f = 0.65; mp = 127.5 °C; 300-MHz ^{1}H NMR ($CDCl_3$) δ 1.11 (d, 3 H, Thr-C^{4}H$_3$, J = 6.4 Hz), 1.15 (d, 3 H, Thr-C^{4}H$_3$, J = 6.4 Hz), 1.45 (s, 9 H, Boc, C(CH$_3$)$_3$), 2.78 (d, 3 H, NHCH_3, J = 4.8 Hz), 3.93 (d, 2 H, Gly-C^{2}H$_2$, J_{HNH} = 5.4 Hz), 4.16 (m, 1 H, Thr-C^{2}H), 4.21 (dq, 1 H, Thr-C^{3}H, J = 2.8 Hz), 4.30 (m, 1 H, Thr-C^{2}H), 4.56 (m, 1 H, Thr-C^{3}H), 4.51, 4.52, 4.53 and 4.59 (4d, 4 H, 2 benzyl, PhCH_2, J = 10.9, 12.3 Hz), 5.51 (d, 1 H, Thr-NH, J = 7.2 Hz), 6.75 (q, 1 H, NHCH$_3$), 6.97 (d, 1 H, Thr-NH, J = 7.4 Hz), 7.30 (m, 11 H, Gly-NH, 2 benzyl, PhCH$_2$, aromatic part); ^{13}C NMR ($CDCl_3$) δ 15.7 and 15.8 (2 Thr-C^4), 26.3 (NHCH$_3$), 28.2 (Boc), 43.6 (Gly-C^2), 56.6 (2 Thr-C^2), 71.5 and 71.7 (2 benzyl, PhCH_2), 73.9 and 74.4 (2 Thr-C^3), 127.7, 127.8, 128.4 and 137.8 (2 benzyl, PhCH$_2$, aromatic part), 168.5, 169.6 and 171.3 (Gly-C^1, 2 Thr-C^1).

Boc-Thr(OH)-Gly-Ser(OH)-NHCH$_3$ (10). To a solution of **7** (1.76 g, 3.17 mmol) in MeOH (30 mL) was added a catalytic amount of 10% Pd/C. The mixture was slowly stirred overnight under H_2 atmosphere (balloon). The reaction mixture was centrifuged, and the supernatant was filtered over a millipore filter (0.2 μm) and concentrated *in vacuo* to give **10** (1.76 g, 3.10 mmol) as a white solid in 98% yield: R_f = 0.31; mp = 84 °C; 300-MHz ^{1}H NMR (CD_3OD) δ 1.19 (d, 3 H, Thr-C^{4}H$_3$, J = 6.4 Hz), 1.43 (s, 9 H, Boc, C(CH$_3$)$_3$), 2.74 (s, 3 H, NHCH_3), 3.69 and 3.80 (8 lines, AB of ABX, 2 H, Ser-C^{3}H$_2$, J_{AX} = 4.6 Hz, J_{BX} = 5.2 Hz, J_{AB} = 11.5 Hz), 3.79 and 4.04 (2d, 2 H, Gly-C^{2}H$_2$, J_{AB} = 16.7 Hz), 4.08 (d, 1 H, Thr-C^{2}H, J = 2.8 Hz), 4.26 (dq, 1 H, Thr-C^{3}H), 4.40 (t, 1 H, Ser-C^{2}H); ^{13}C NMR (CD_3OD) δ 19.9 (Thr-C^4), 26.4 (NHCH$_3$), 28.6 and 81.0 (Boc), 43.9 (Gly-C^2), 56.8 (Ser-C^2), 61.6 (Thr-C^2), 62.8 (Ser-C^3), 68.6 (Thr-C^3), 171.7, 172.5 and 174.4 (Ser-C^1, Gly-C^1, Thr-C^1).

Boc-Ser(OH)-Gly-Ser(OH)-NHCH$_3$ (11). Compound **8** (1.59 g, 2.93 mmol) in MeOH was treated under the same conditions employed for the hydrogenolysis of compound **7**. A white solid (1.01 g, 2.78 mmol) was obtained in 95% yield: R_f = 0.20; mp = 144.5 °C; 300-MHz ^{1}H NMR (CD_3OD) δ 1.45 (s, 9 H, Boc, C(CH$_3$)$_3$), 2.74 (s, 3 H, NHCH_3), 3.74 and 3.82 (8 lines, AB of ABX, 4 H, 2 Ser-C^{3}H$_2$, J_{AX} = 5.3 Hz, J_{BX} = 5.2 Hz, J_{AB} = 11.0 Hz), 3.79 and 3.96 (2d, 2 H, Gly-C^{2}H$_2$, J_{AB} = 16.6 Hz), 4.13 (t, 1 H, Ser-C^{2}H, J_{vic} = 5.1 Hz), 4.38 (t, 1 H, Ser'-C^{2}H, J_{vic} = 5.3 Hz); ^{13}C NMR (CD_3OD) δ 26.4 (NHCH$_3$), 28.6 (Boc), 44.0 (Gly-C^2), 56.9 and 58.3 (2 Ser-C^2), 62.8 and 63.3 (2 Ser-C^3).

Boc-Thr(OH)-Gly-Thr(OH)-NHCH$_3$ (12). Hydrogenolysis of compound **9** (1.89 g, 3.31 mmol) was carried out according to the same conditions employed for the hydrogenolysis of compound **7**. A white solid (1.12 g, 3.11 mmol) was obtained in 94% yield: R_f = 0.15; mp = 167 °C; 300-MHz ^{1}H NMR (CD_3OD) δ 1.14 (d, 3 H, Thr-C^{4}H$_3$, J = 6.3 Hz), 1.20 (d, 3 H, Thr-C^{4}H$_3$, J = 6.4 Hz), 1.46 (s, 9 H, Boc, C(CH$_3$)$_3$), 2.74 (s, 3 H, NHCH_3), 3.88 and 4.05 (2d, 2 H, Gly-C^{2}H$_2$, J_{AB} = 16.7 Hz), 4.05 (d, 1 H, Thr-C^{2}H, J = 2.8 Hz), 4.24 (m, 3 H, Thr-C^{2}H, 2 Thr-C^{3}H); ^{13}C NMR (CD_3OD) δ 20.0 and 20.2 (2 Thr-C^4), 26.4 (NHCH$_3$), 28.7 and 80.9 (Boc), 43.9 (Gly-C^2), 60.1 and 61.5 (2 Thr-C^2), 68.0 and 68.5 (2 Thr-C^3), 171.8, 172.9 and 174.5 (Gly-C^1, 2 Thr-C^1).

4-Chlorobenzyl Dichlorophosphinite (14). To a solution of PCl_3 (8.55 mL, 98.00 mmol) and pyridine (7.93 mL, 98.00 mmol) in dry ether (200 mL) under argon was added dropwise over a period of 45 min at −60 °C (ethanol/CO_2) a solution of 4-chlorobenzyl alcohol (14.26 g, 0.1 mol) in dry ether (50 mL). After 45 min the reaction mixture was kept at rt for 1 h. After the precipitate was filtered off under argon, the solvent was evaporated *in vacuo*, resulting in a pale yellow oil. Distillation *in vacuo* gave a colorless oil: bp = 120–123 °C (0.5 mmHg); ^{31}P NMR 177.9.

Phosphate-Protected Cyclic Phosphopeptide 15. Method A. Compound **10** (287 mg, 0.76 mmol) and 1*H*-tetrazole (111 mg, 1.58 mmol) were coevaporated with dry dioxane (5 × 5 mL) and subsequently dissolved in dry CH_3CN (15.25 mL), yielding a 50 mM solution of the starting material. Under argon a 0.15 M solution of freshly prepared 4-chlorobenzyl bis(*N*,*N*-diisopropylamino)phosphinite[19] (**13**) in dry CH_3CN (10.0 mL) was added dropwise (0.4 mL/h). After 30 h the phosphite triester (R_f = 0.37) was oxidized with 80% *tert*-butyl hydroperoxide (0.38 mL, 3.04 mmol) in 2 h at rt. A solution of 10% aqueous $NaHSO_3$ (25 mL) was then added, and the reaction mixture was concentrated *in vacuo* after 10 min. EtOAc (50 mL) was added to the residue, and the organic layer was washed with 10% aqueous $NaHSO_3$ (2 × 25 mL) and brine (25 mL). After purification by flash chromatography compound **15** (**15a**: 129 mg, 0.23 mmol, **15b**: 8.5 mg, 15 μmol) was obtained as a white solid in 32% total yield. The diastereoisomers could be separated and were obtained in a ratio of 15:1: R_f(**15a**) = 0.26, R_f (**15b**) = 0.30.

Method B. Compound **10** (377 mg, 1.00 mmol) was coevaporated with dry dioxane (5 × 5 mL) and subsequently dissolved in dry CH_2Cl_2/CH_3CN (2/1, v/v, 20.0 mL) resulting in a 50 mM solution of the starting material. Under argon, diisopropylethylamine (DIPEA) (375 μL, 2.20 mmol) and 4-chlorobenzyl dichlorophosphinite (**14**, 120 μL, 1.16 mmol) were added successively. The reaction mixture was stirred for 3 h at ambient temperature. Oxidation of the obtained phosphite triester to the corresponding phosphate triester was carried out by addition of 3-chloroperbenzoic acid (55%, 631 mg, 2.01 mmol) at 0 °C, followed by stirring for 20 min at rt. Compound **15**, isolated by the workup procedure as described for method A, was obtained as a white solid in 63% total yield. The diastereoisomers could be separated and were obtained in a ratio of 4.2:1 (**15a**: 290 mg, 0.50 mmol, **15**: 70 mg, 0.12 mmol). NMR data of diastereoisomer **15a**: 300-MHz ^{1}H NMR (CD_3OD) δ 1.35 (d, 3 H, Thr-C^{4}H$_3$, J = 6.4 Hz), 1.52 (s, 9 H, Boc, C(CH$_3$)$_3$), 2.78 (s, 3 H, NHCH_3), 3.80 and 4.04 (2d, 2 H, Gly-C^{2}H$_2$, J_{AB} = 16.6 Hz), 4.28 and 4.42 (16 lines, AB of ABX, 2 H, Ser-C^{3}H$_2$, J_{AX} = J_{BX} = 2.8 Hz, J_{AB} = 11.2 Hz, J_{AP} = 10.3 Hz, J_{BP} = 10.8 Hz), 4.42 (t, 1 H, Thr-C^{2}H, J_{vic} = J_{HP} = 2.1 Hz), 4.73 (q, 1 H, Ser-C^{2}H, J_{HP} = 2.8 Hz), 5.08 (d, 2 H, 4-chlorobenzyl, PhCH_2, J_{HP} = 8.8 Hz), 5.32 (10 lines, 1 H, Thr-C^{3}H, J_{HP} = 6.4 Hz), 7.40 (s, 4 H, 4-chlorobenzyl, PhCH$_2$, aromatic part); ^{13}C NMR (CD_3OD) δ 18.0 (Thr-C^4), 26.6 (NHCH$_3$), 28.6, 81.4 and 157.7 (Boc), 44.6 (Gly-C^2), 53.3 (Ser-C^2, J_{CP} = 4.4 Hz), 60.1 (Thr-C^2, J_{CP} = 10.3 Hz), 69.8 (4-chlorobenzyl, PhCH_2, J_{CP} = 5.9 Hz), 70.3 (Ser-C^3, J_{CP} = 5.9 Hz), 79.6 (Thr-C^3, J_{CP} = 4.4 Hz), 129.7, 130.8, 135.5 and 135.8 (4-chlorobenzyl, PhCH$_2$, aromatic part), 170.6, 171.2 and 172.7 (Ser-C^1, Gly-C^1, Thr-C^1); ^{31}P NMR δ −0.15. NMR data of diastereoisomer **15b**: 300-MHz ^{1}H NMR (CD_3OD) δ 1.39 (d, 3 H, Thr-C^{4}H$_3$, J = 6.4 Hz), 1.52 (s, 9 H, Boc, C(CH$_3$)$_3$), 2.77 (s, 3 H, NHCH_3), 3.80 and 4.03 (2d, 2 H, Gly-C^{2}H$_2$, J_{AB} = 16.4 Hz), 4.14 and 4.29 (16 lines, AB of ABX, 2 H, Ser-C^{3}H$_2$, J_{AX} = 2.9 Hz, J_{BX} = 5.6 Hz, J_{AB} = 11.2 Hz, J_{AP} = 8.3 Hz, J_{BP} = 9.5 Hz), 4.36 (dd, 1 H, Thr-C^{2}H, J_{vic} = 2.3 Hz, J_{HP} = 4.3 Hz), 4.59 (d, 1 H, Ser-C^{2}H), 5.09 (m, 1 H, Thr-C^{3}H), 5.19 (d, 2 H, 4-chlorobenzyl, PhCH_2, J_{HP} = 9.4 Hz), 7.46 (s, 4 H, 4-chlorobenzyl, PhCH$_2$, aromatic part); ^{13}C NMR (CD_3OD) δ 17.9 (Thr-C^4), 26.5 (NHCH$_3$), 28.6, 81.5 (Boc), 44.6 (Gly-C^2), 54.1 (Ser-C^2, J_{CP} = 5.9 Hz), 60.2 (Thr-C^2, J_{CP} = 8.6 Hz), 70.0 (4-chlorobenzyl, PhCH_2, J_{CP} = 7.3 Hz), 70.7 (Ser-C^3, J_{CP} = 5.9 Hz), 80.4 (Thr-C^3, J_{CP} = 5.9 Hz), 130.0, 131.2 and 135.4 (4-chlorobenzyl, PhCH$_2$, aromatic part), 170.5, 171.7 and 172.8 (Ser-C^1, Gly-C^1, Thr-C^1); ^{31}P NMR δ −4.31; MS (FAB) *m/e* 585 (M + Na)$^+$, 563 (M + H)$^+$.

Phosphate-Protected Cyclic Phosphopeptide 16. Method A. To a solution of compound **11** (438 mg, 1.21 mmol) and 1*H*-tetrazole (175 mg, 2.50 mmol) in dry CH_3CN was added dropwise a solution of 4-chlorobenzyl bis(*N*,*N*-diisopropylamino)-phosphinite (**13**) in dry CH_3CN, according to procedure A for the preparation of compound **15**. Purification by flash chromatography gave compound **16** (**16a**: 100 mg, 0.18 mmol, **16b**: 108 mg, 0.20 mmol) as a white solid in 31% total yield. The diastereoisomers could be separated and were obtained in a ratio of 1:1: R_f(**16a**) = 0.39, R_f(**16b**) = 0.45.

Method B. Compound **11** (363 mg, 1.00 mmol), DIPEA (375 μL, 2.21 mmol), and 4-chlorobenzyl dichlorophosphinite (**14**, 120 μL, 1.16 mmol) in dry CH_2Cl_2/CH_3CN were used to prepare **16** according to procedure B described for the preparation of compound **15**. After purification by flash chromatography compound **16** (**16a**: 120 mg, 0.22 mmol, **16b**: 267 mg, 0.49 mmol) was isolated as a white solid in 70% total yield. The diastereoisomers could be separated and were obtained in a ratio of 2.2:1. NMR data of diastereoisomer **16a**: 300-MHz ^{1}H NMR (CD_3OD)

δ 1.44 (s, 9 H, Boc, C(CH$_3$)$_3$), 2.75 (s, 3 H, NHCH_3), 3.79 and 4.09 (2d, 2 H, Gly-C^{2}H$_2$, J_{AB} = 18.1 Hz), 4.03 (m, 1 H, Ser-C^{3}H$_a$), 4.38 (m, 4 H, Ser-C^{3}H$_b$, Ser-C^{3}H$_2$, Ser-C^{2}H), 4.76 (m, 1 H, Ser-C^{2}H), 5.03 (d, 2 H, 4-chlorobenzyl, PhCH_2, J_{HP} = 8.9 Hz), 7.42 (s, 4 H, 4-chlorobenzyl, PhCH$_2$, aromatic part); ^{13}C NMR (CDCl$_3$) δ 26.2 (NHCH$_3$), 28.2 and 155.1 (Boc), 44.0 (Gly-C^2), 53.1 (2 Ser-C^2), 69.8 (2 Ser-C^3, 4-chlorobenzyl, PhCH_2), 129.0, 129.6, 133.3 and 135.1 (4-chlorobenzyl, PhCH$_2$, aromatic part), 168.7 and 170.6 (2 Ser-C^1, Gly-C^1); ^{31}P NMR δ-2.39. NMR data of diastereoisomer **16b**: 300-MHz ^{1}H NMR (CD$_3$OD) δ 1.45 (s, 9 H, Boc, C(CH$_3$)$_3$), 2.74 (s, 3 H, NHCH_3), 3.91 (m, 2 H, Ser-C^{3}H$_2$), 3.87 and 4.01 (2d, 2 H, Gly-C^{2}H$_2$, J_{AB} = 16.6 Hz), 4.38 (m, 4 H, Ser-C^{3}H$_2$, 2 Ser-C^{2}H), 5.03 (d, 2 H, 4-chlorobenzyl, PhCH$_2$, J_{HP} = 8.6 Hz), 7.33 (s, 4 H, 4-clorobenzyl, PhCH$_2$, aromatic part); ^{31}P NMR δ −0.45; MS (FAB) *m/e* 571 (M + Na)$^+$, 549 (M + H)$^+$.

Phosphate-Protected Cyclic Phosphopeptide 17. Method A. To a solution of compound **12** (326 mg, 0.83 mmol) and 1*H*-tetrazole (139 mg, 1.98 mmol) in dry CH$_3$CN was added dropwise a solution of 4-chlorobenzyl bis(*N,N*-diisopropylamino)phosphinite (**13**) in dry CH$_3$CN, according to procedure A for the preparation of compound **15**. Purification by flash chromatography gave compound **17** (154 mg, 0.27 mmol) as a white solid in 32% total yield. The diastereoisomers, which could not be separated, were obtained in a ratio of 3:1 as was estimated from ^{31}P NMR: R_f = 0.35.

Method B. Compound **12** (390 mg, 1.00 mmol), DIPEA (375 mL, 2.21 mmol), and 4-chlorobenzyl dichlorophosphinite (**14**, 120 μL, 1.16 mmol) in dry CH$_2$Cl$_2$/CH$_3$CN were used to prepare **17** according to procedure B for the preparation of compound **15**. Compound **17** (356 mg, 0.62 mmol) was isolated, after purification by flash chromatography, as a white solid in 62% yield. The diastereoisomers, which could not be separated, were obtained in a ratio of 1:1 as was estimated from ^{31}P NMR. NMR data of diastereoisomer **17a**: 300-MHz ^{1}H NMR (CD$_3$OD) δ 1.34 (d, 6 H, 2 Thr-C^{4}H$_3$, J = 6.4 Hz), 1.50 (s, 9 H, Boc, C(CH$_3$)$_3$), 2.72 (s, 3 H, NHCH_3), 3.70 and 4.04 (2d, 2 H, Gly-C^{2}H$_2$, J_{AB} = 16.8 Hz), 4.35 (dd, 1 H, Thr-C^{2}H, J_{vic} = 6.5 Hz, J_{HP} = 1.2 Hz), 4.42 (dd, 1 H, Thr′-C^{2}H, J_{vic} = 2.5 Hz, J_{HP} = 4.0 Hz), 4.65 (16 lines, 1 H, Thr-C^{3}H, J_{HP} = 9.9 Hz), 5.04 and 5.08 (2 dd, 2 H, 4-chlorobenzyl, PhCH_2, J_{AB} = 12.0 Hz, J_{HP} = 8.2 Hz), 5.32 (10 lines, 1 H, Thr′-C^{3}H, J_{HP} = 6.1 Hz), 7.40 (s, 4 H, 4-chlorobenzyl, PhCH$_2$, aromatic part); ^{13}C NMR (CD$_3$OD) δ 18.2 and 18.6 (2 Thr-C^4), 26.4 (NHCH$_3$), 28.4 and 81.4 (Boc), 44.4 (Glyc-C^2), 58.9 (Thr-C^2, J_{CP} = 6.9 Hz), 60.1 (Thr-C^2), 69.7 (4-chlorobenzyl, PhCH_2, J_{CP} = 7.3 Hz), 78.4 (Thr-C^3, J_{CP} = 8.8 Hz), 79.8 (Thr-C^3, J_{CP} = 4.4 Hz), 129.8 and 130.6 (4-chlorobenzyl, PhCH$_2$, aromatic part), 171.4 and 172.7 (Gly-C^1, 2 Thr-C^1); ^{31}P NMR δ −4.71. NMR data of diastereomer **17b**: 300-MHz ^{1}H NMR (CD$_3$OD) δ 1.33 (d, 6 H, 2 Thr-C^{4}H$_3$, J = 6.2 Hz), 1.46 (s, 9 H, Boc, C(CH$_3$)$_3$), 2.74 (s, 3 H, NHCH_3), 3.78 and 4.02 (2d, 2 H, Gly-C^{2}H$_2$, J_{AB} = 16.8 Hz), 4.26 (t, 1 H, Thr-C^{2}H, J_{vic} = J_{HP} = 9.9 Hz), 4.41 (m, 2 H, Thr-C^{2}H, Thr-C^{3}H), 4.73 (m, 1 H, Thr-C^{3}H), 5.03 (d, 2 H, 4-chlorobenzyl, PhCH_2, J_{HP} = 8.8 Hz), 7.34 (s, 4 H, 4-chlorobenzyl, PhCH$_2$, aromatic part); ^{31}P NMR δ −1.53; MS (FAB) *m/e* 599 (M + Na)$^+$, 577 (M + H)$^+$.

Cyclic Phosphopeptide 18. To a solution of compound **15** (100 mg, 0.18 mmol) in 2-methyl-2-propanol (*t*-BuOH)/H$_2$O (4/1, v/v, 10 mL) were added sodium acetate (28 mg, 0.21 mmol) and a catalytic amount of 10% Pd/C. The reaction mixture was slowly stirred under H$_2$ atmosphere (balloon) for 1 h at rt. The reaction mixture was centrifuged, and the supernatant was filtered over a millipore filter (0.2 μm) and concentrated *in vacuo*. Purification on Sephadex LH-20 (100% MeOH) and subsequent lyophilization gave compound **18** (78 mg, 0.17 mmol) as a white solid in 95% yield. Compound **18** was pure on TLC (RP-2, R_f = 0.76) and according to NMR: 600-MHz ^{1}H NMR (D$_2$O) δ 1.45 (d, 3 H, Thr-C^{4}H$_3$, J = 6.7 Hz), 1.45 (s, 9 H, Boc, C(CH$_3$)$_3$), 2.74 (s, 3 H, NHCH_3), 3.80 and 4.14 (2d, 2 H, Gly-C^{2}H$_2$, J_{AB} = 17.4 Hz), 4.01 and 4.28 (16 lines, AB of ABX, 2 H, Ser-C^{3}H$_2$, J_{AX} = 3.0 Hz, J_{BX} = 2.7 Hz, J_{AB} = 11.6 Hz, J_{AP} = 10.6 Hz, J_{BP} = 7.1 Hz), 4.15 (dd, 1 H, Thr-C^{2}H, J_{vic} = 2.6 Hz, J_{HP} = 3.8 Hz), 4.43 (t, 1 H, Ser-C^{2}H), 4.85 (tq, 1 H, Thr-C^{3}H, J_{HP} = 2.3 Hz); ^{13}C NMR (CD$_3$OD) δ 18.4 (Thr-C^4), 26.6 (NHCH$_3$), 28.6, 81.2 and 157.9 (Boc), 44.1 (Gly-C^2), 55.1 (Ser-C^2, J_{CP} = 4.4 Hz), 61.4 (Thr-C^2, J_{CP} = 8.8 Hz), 67.1 (Ser-C^3, J_{CP} = 5.9 Hz), 75.8 (Thr-C^3, J_{CP} = 4.4 Hz), 171.5, 171.6 and 174.2 (Ser-C^1, Gly-C^1, Thr-C^1); ^{31}P NMR δ −0.39; 500-MHz ^{1}H NMR (DMSO-d_6) δ 1.21 (Thr-C^{4}H$_3$), 1.42 (Boc, C(CH$_3$)$_3$), 2.58 (NHCH_3, J_{HNH} = 4.2 Hz), 3.56 (Gly-C^{2}H$_a$(*pro-S*), J_{HNH} = 4.4 Hz, J_{AB} = 17.4 Hz), 3.88 (Gly-C^{2}H$_b$(*pro-R*), J_{HNH} = 8.3 Hz), 3.88 (Ser-C^{3}H$_a$(*pro-S*)), 3.95 (Ser-C^{3}Hb(*pro-R*)), 3.95 (Thr-C^{2}H, J_{vic} = 2.9 Hz, J_{HNH} = 9.5 Hz), 4.01 (Ser-C^{2}H, J_{HNH} = 7.9 Hz), 4.55 (Thr-C^{3}H), 6.52 (Thr-NH), 7.43 (Ser-NH, J_{NH-C^3} = 1.8 Hz), 7.69 (N*H*CH$_3$), 8.52 (Gly-NH); ^{13}C NMR (DMSO-d_6) δ 18.5 Thr-C^4), 25.8 (NHCH$_3$), 27.9, 79.0 and 155.6 (Boc), 43.4 (Gly-C^2), 54.1 (Ser-C^2), 59.9 (Thr-C^2), 65.6(Ser-C^3), 72.7 (Thr-C^3), 168.8 (Gly-C^1), 169.8 (Ser-C^1), 171.5 (Thr-C^1); MS (FAB) *m/e* 483 (M + Na)$^+$, 461 (M + H)$^+$.

Cyclic Phosphopeptide 19. Compound **16** (57 mg, 0.10 mmol), sodium acetate (21 mg, 0.16 mmol), and 10% Pd/C were suspended in *t*-BuOH/H$_2$O (4/1, v/v, 10 mL), and the mixture was treated according to the procedure for the preparation of compound **18**. The reaction afforded, after purification on Sephadex LH-20 (100% MeOH) and lyophilization, a white solid (43 mg, 96 μmol) in 92% yield. Compound **19** was pure on TLC (RP-2, R_f = 0.93) and according to NMR: 300-MHz ^{1}H NMR (CD$_3$OD) δ 1.48 (s, 9 H, Boc, C(CH$_3$)$_3$), 2.76 (s, 3 H, NHCH_3), 3.70 and 4.18 (2d, 2 H, Gly-C^{2}H$_2$, J_{AB} = 17.2 Hz), 4.04 and 4.35 (12 lines, AB of ABX, 2 H, Ser′-C^{3}H$_2$, J_{AX} = J_{AP} = 2.9 Hz, J_{BX} = J_{BP} = 3.1 Hz, J_{AB} = 9.9 Hz), 4.15 and 4.33 (12 lines, AB of ABX, 2 H, Ser-C^{3}H$_2$, J_{AX} = 2.7 Hz, J_{BX} = 2.2 Hz, J_{BP} = 9.4 Hz, J_{AB} = J_{AP} = 11.4 Hz), 4.21 (q, 1 H, Ser′-C^{2}H, J_{vic} = J_{HP} = 2.8 Hz), 4.26 (t, 1 H, Ser-C^{2}H, J_{vic} = 2.4 Hz); 75-MHz ^{13}C NMR (CD$_3$OD) δ 26.5 (NHCH$_3$), 28.7 and 81.2 (Boc), 43.9 (Gly-C^2), 55.7 (Ser-C^2), 57.4 (d, Ser′-C^2, J_{CP} = 10.3 Hz), 67.5 (d, Ser-C^3, J_{CP} = 4.9 Hz), 68.1 (Ser′-C^3), 171.4, 171.9 and 174.1 (2 Ser-C^1, Gly-C^1); ^{31}P NMR δ 0.60; MS (FAB) *m/e* 469 (M + Na)$^+$, 447 (M + H)$^+$.

Cyclic Phosphopeptide 20. Compound **17** (162 mg, 0.28 mmol), sodium acetate (70 mg, 0.51 mmol), and 10% Pd/C were dissolved or suspended in *t*-BuOH/H$_2$O (4/1, v/v, 10 mL), and the mixture was treated according to the same conditions as employed for the preparation of **18**. The reaction afforded, after purification on Sephadex LH-20 (100% MeOH) and lyophilization, a white crystalline material (124 mg, 0.26 mmol) in 93% yield. Compound **20** was pure on TLC (RP-2, R_f = 0.86) and according to NMR: 300-MHz ^{1}H NMR (CD$_3$OD) δ 1.29 (d, 3 H, Thr-C^{4}H$_3$, J = 6.2 Hz), 1.42 (d, 3 H, Thr′-C^{4}H$_3$, J = 6.3 Hz), 1.50 (s, 9 H, Boc, C(CH$_3$)$_3$), 2.73 (s, 3 H, NHCH_3), 3.59 and 4.16 (2d, 2 H, Gly-C^{2}H$_2$, J_{AB} = 16.9 Hz), 3.85 (dd, 1 H, Thr-C^{2}H, J_{vic} = 9.9 Hz, J_{HP} = 1.6 Hz), 4.20 (t, 1 H, Thr′-C^{2}H, J_{vic} = J_{HP} = 1.8 Hz), 4.31 (10 lines, 1 H, Thr-C^{3}H, J_{HP} = 3.4 Hz), 4.95 (16 lines, 1 H, Thr′-C^{3}H, J_{HP} = 2.4 Hz); ^{13}C NMR (CD$_3$OD) δ 18.7 (Thr′-C^4), 19.6 (Thr-C^4), 26.4 (NHCH$_3$), 28.7, 81.3 and 158.1 (Boc), 43.8 (Gly-C^2), 61.2 (Thr′-C^2, J_{CP} = 8.9 Hz), 61.7 (Thr-C^2, J_{CP} = 4.4 Hz), 73.1 (Thr-C^3, J_{CP} = 5.2 Hz), 75.6 (Thr′-C^3), 171.5, 172.3 and 173.8 (Gly-C^1, 2 Thr-C^1); ^{31}P NMR δ −1.39; MS (FAB) *m/e* 497 (M + Na)$^+$, 475 (M + H)$^+$.

Deprotected Cyclic Phosphopeptide 21. To a solution of compound **15** (93 mg, 0.17 mmol) in *t*-BuOH/H$_2$O (4/1, v/v, 10 mL) was added a catalytic amount of 10% Pd/C. The reaction mixture was slowly stirred under H$_2$ atmosphere (balloon) over night at rt. The reaction mixture was centrifuged, and the supernatant was filtered over a millipore filter (0.2 μm) and concentrated *in vacuo*. The residue was dissolved in CH$_2$Cl$_2$ (2.5 mL) and cooled to 0 °C, and TFA (2.5 mL) was added. The reaction mixture was stirred for 3 h at 0 °C, concentrated *in vacuo*, and coevaporated with ether (5 × 2 mL). The residue was dissolved in H$_2$O (2.5 mL), neutralized with 0.1 M aqueous NH$_4$HCO$_3$, and concentrated *in vacuo*. Purification on Sephadex LH-20 (MeOH/H$_2$O, 85/15, v/v) and subsequent lyophilization afforded compound **21** (51 mg, 0.15 mmol) as a white solid in 96% yield. Compound **21** was pure according to NMR: 300-MHz ^{1}H NMR (D$_2$O) δ 1.54 (d, 3 H, Thr-C^{4}H$_3$, J = 6.5 Hz), 2.76 (s, 3 H, NHCH_3), 3.95 and 4.10 (2d, 2 H, Gly-C^{2}H$_2$, J_{AB} = 16.7 Hz), 4.08 (t, 1 H, Thr-C^{2}H, J_{vic} ≈ J_{HP} ≈ 2.0 Hz), 4.21 (m, 2 H, Ser-C^{3}H$_2$), 4.40 (t, 1 H, Ser-C^{2}H, J_{vic} = 3.3 Hz), 4.87 (m, 1 H, Thr-C^{3}H); ^{13}C NMR (D$_2$O) δ 17.7 (Thr-C^4), 26.3 (NHCH$_3$), 43.7 (Gly-C^2), 54.6 (Ser-C^2, J_{CP} = 2.9 Hz), 58.2 (Thr-C^2, J_{CP} = 10.3 Hz), 66.8 (Ser-C^3, J_{CP} = 5.9 Hz), 73.7 (Thr-C^3, J_{CP} = 4.4 Hz), 171.2 and 171.5 (Ser-C^1, Gly-C^1, Thr-C^1); ^{31}P NMR δ −0.03; MS (FAB) *m/e* 377 (M + K)$^+$, 361 (M + Na)$^+$, 339 (M + H)$^+$.

Deprotected Cyclic Phosphopeptide 22. Compound **16** (108 mg, 0.20 mmol) was deprotected by treatment according to the

Table IV. ^1H and ^{13}C NMR Chemical Shifts δ (ppm) of 18 in DMSO-d_6 at 300 K

residue	proton	δ	carbon	δ
Thr	HN	6.52		
	C^2H	3.95	C^2	59.9
	C^3H	4.55	C^3	72.7
	C^4H_3	1.21	C^4	18.5
			C^1	171.5
Gly	HN	8.52		
	C^2H_a (*pro-S*)	3.56	C^2	43.4
	C^2H_b (*pro-R*)	3.88		
			C^1	168.8
Ser	HN	7.43		
	C^2H	4.01	C^2	54.1
	C^3H_a (*pro-S*)	3.88	C^3	65.6
	C^3H_b (*pro-R*)	3.95		
			C^1	169.8
Boc	Me	1.42	$C(CH_3)_3$	27.9
			$C(CH_3)_3$	79.0
			C(O)O	155.6
$NHCH_3$	HN	7.69		
	Me	2.58	CH_3	25.8

same conditions employed for the preparation of **21**. After purification on Sephadex LH-20 ($MeOH/H_2O$, 85/15, v/v) and lyophilization compound **22** was obtained as a white crystalline material (57 mg, 0.17 mmol) in 90% yield. Compound **22** was pure according to NMR: 300-MHz ^1H NMR (D_2O) δ 2.77 (s, 3 H, $NHCH_3$), 3.77 and 4.42 (2d, 2 H, Gly-C^2H_2, J_{AB} = 17.0 Hz), 4.30 (m, 3 H, Ser'-C^3H_a, Ser-C^3H_2), 4.39 (m, 1 H, Ser-C^2H), 4.52 (m, 2 H, Ser'-C^2H, Ser'-C^3H_b); 75-MHz ^{13}C NMR (D_2O) δ 27.2 ($NHCH_3$), 44.3 (Gly-C^2), 54.2 (Ser'-C^2, J_{CP} = 10.6 Hz), 55.8 (Ser-C^2), 65.8 (Ser-C^3), 67.8 (Ser'-C^3, J_{CP} = 5.6 Hz), 169.6, 171.9 and 172.5 (2 Ser-C^1, Gly-C^1); ^{31}P NMR δ −0.80; MS (FAB) *m/e* 369 $(M - H + 2Na)^+$, 347 $(M + Na)^+$, 325 $(M + H)^+$.

Deprotected Cyclic Phosphopeptide 23. Compound **20** (114 mg, 0.20 mmol) was deprotected by treatment according to the procedure for the preparation of compound **21**. After purification on Sephadex LH-20 ($MeOH/H_2O$, 85/15, v/v) and lyophilization compound **23** (63 mg, 0.18 mmol) was isolated as a white crystalline material in 90% yield. Compound **23** was pure according to NMR: 300-MHz ^1H NMR (CD_3OD) δ 1.29 (d, 3 H, Thr-C^4H_3, J = 6.1 Hz), 1.48 (d, 3 H, Thr-C^4H_3, J = 6.4 Hz), 2.72 (s, 3 H, $NHCH_3$), 3.69 (m, 2 H, 2 Thr-C^2H), 3.78 and 4.00 (2d, 2 H, Gly-C^2H_2, J_{AB} = 17.9 Hz), 4.27 (2 Thr-C^3H); ^{13}C NMR (D_2O) δ 17.7, 18.9 (2 Thr-C^4), 26.4 ($NHCH_3$), 43.1 (Gly-C^2), 59.1 (Thr-C^2, J_{CP} = 10.3 Hz), 60.9 (Thr-C^2, J_{CP} = 4.4 Hz), 72.8 (Thr-C^3, J_{CP} = 5.9 Hz), 75.8 (Thr-C^3, J_{CP} = 4.4 Hz), 171.8, 175.9 (Gly-C^1, 2 Thr-C^1); ^{31}P NMR δ −1.99; MS (FAB) *m/e* 413 $(M - H + Na + K)^+$, 397 $(M - H + 2Na)^+$, 391 $(M + K)^+$, 375 $(M + Na)^+$, 353 $(M + H)^+$.

B. Structure. NMR Spectroscopy. Two-dimensional spectra were acquired with quadrature detection, TPPI,[30] in both dimensions. Methyl group- and t_1 ridges were minimized with AURELIA software. A sample containing 9.5 mg/0.5 mL of cyclic phosphopeptide **18** in degassed dimethyl sulfoxide-d_6, which corresponds to a concentration of 41.3 mmol/L, was used for all NMR measurements.

^1H-resonances of the compound could be assigned with TOCSY[31,32] and DQF-COSY[33] techniques. For the assignment of H-bearing carbon atoms, the ^1H-detected heteronuclear shift-correlation experiment HMQC[34-36] was used (Table IV).

Determination of $^3J(NH,C^2H)$, if they could not be extracted from 1D spectra, and $^3J(C^2H,C^3H)$ was carried out with the DISCO procedure.[37-39] Heteronuclear long-range couplings $^3J(NH,C^2)$ were determined by a TOCSY with ω_1 half-filter.[40-42] Qualitative determination of heteronuclear couplings was possible by use of a heteronuclear multiple bond correlation, HMBC.[43]

Proton–proton distances for conformation analysis were achieved by quantitative evaluation of a ROESY spectrum[44,45] with a mixing time of 120 ms and a spin lock field of 4 kHz. The integrals were offset corrected,[46] and for calibration 240 pm was used for the distance between Thr-C^3H and Thr-C^4H_3. All 2D-spectra are processed with a 90° shifted square sinebell function in both dimensions.

TOCSY (HOHAHA): spectral width 12.496 ppm, relaxation delay 1.3 s, time domain 4096, eight scans, 512 increments, trim pulse 2.5 ms, mixing time (MLEV-17) 67.9 ms.

TOCSY with ω_1 half-filter and BIRD presaturation: spectral width 12.496 ppm, relaxation delay 100 ms, time domain 4096, 80 scans, 512 increments, BIRD pulse recovery delay 198 ms.

Doublequantum filtered COSY (DQF-COSY): spectral width 12.496 ppm, relaxation delay 1.3 s, time domain 8192, 32 scans, 256 increments.

ROESY: spectral width 12.496 ppm, relaxation delay 1.3 s, time domain 4096, 80 scans, 496 increments, 4 kHz spin lock field, mixing time 120 ms.

^1H, ^{13}C-Heteronuclear multiquantum coherence with BIRD presaturation (BIRD-HMQC): spectral width 12.496 ppm, 79.513 ppm in the ^{13}C dimension, relaxation delay 1.3 s, time domain 8192, 48 scans, 256 increments, BIRD pulse recovery delay 198 ms.

^1H,^{13}C-Heteronuclear multiple bond correlation (HMBC): spectral width 12.496 ppm, relaxation delay 1.3 s, time domain 8192, 160 scans, 512 increments. Increased resolution in t_1 and shorter measuring times were achieved by folding into a spectral range from 15–80 ppm. The first incremental delay in t_1 was adjusted for a phase correction of 180° (0th order), and −360° (1st order).[47] The spectrum was recorded and processed phase sensitive, followed by a magnitude calculation in t_2.

Restrained Molecular Dynamics. The molecular dynamics simulations were carried out with the Discover (BIOSYM) program. The starting structure was built interactively with the INSIGHT program with the backbone dihedral angles (ϕ, ψ, ω) set to 180°. The structure was cyclized by connecting the phosphate group and Ser-C^3 and energy minimization. All computer simulations were carried out on SiliconGraphics 4D/240SX and 4D/70GTB computers.

The simulations were carried out in DMSO following the procedures previously described.[48] The solvent is simulated as four atoms, using a united atom approach for the methyl groups. After partial minimization of the hand-built structure (200 steps of steepest descent), the peptide was placed in the middle of a box of DMSO with dimensions of 3.3 × 2.8 × 2.7 nm containing 166 solvent molecules. The distance restraints (described below) were then applied and the system partially minimized (200 steps of steepest descent).

The distance restraints were derived from the ROEs using the two-spin approximation as discussed above. The upper and lower distance bounds were set to ±5% of the calculated distances, respectively. This small variation allows for some error in the measurement of the intensity of cross peaks and the conversion to distances. For restraints to methyl group protons, the carbon atom was used with the addition of 30 pm to the upper bound,[49]

(30) Marion, D.; Wüthrich, K. *Biochem. Biophys. Res. Commun.* **1983**, *113*, 967.
(31) Braunschweiler, L.; Ernst, R. R. *J. Magn. Reson.* **1983**, *53*, 521.
(32) Davis, D. G.; Bax, A. *J. Am. Chem. Soc.* **1985**, *107*, 2820.
(33) Piantini, U.; Sørensen, O. W.; Ernst, R. R. *J. Am. Chem. Soc.* **1982**, *104*, 6800.
(34) Müller, L. *J. Am. Chem. Soc.* **1979**, *101*, 4481.
(35) Bendall, M. R.; Pegg, D. T.; Dorell, D. M. *J. Magn. Reson.* **1983**, *52*, 81.
(36) Bax, A.; Griffey, R. H.; Hawkins, B. L. *J. Magn. Reson.* **1983**, *55*, 301.
(37) Oschkinat, H.; Freeman, R. *J. Magn. Reson.* **1984**, *60*, 164.
(38) Kessler, H.; Müller, A.; Oschkinat, H. *Magn. Reson. Chem.* **1985**, *23*, 844.
(39) Kessler, H.; Oschkinat, H. *Angew. Chem.* **1985**, *97*, 689; *Angew. Chem., Int. Ed. Engl.* **1985**, *24*, 690.
(40) Schmieder, P.; Kurz, M.; Kessler, H. *J. Biomol. NMR* **1991**, *1*, 403.
(41) Kurz, M.; Schmieder, P.; Kessler, H. *Angew. Chem.* **1991**, *103*, 1341; *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 1329.
(42) Schmieder, P.; Kessler, H. *Biopolymers* **1992**, *32*, 435.
(43) Bax, A.; Summers, M. F. *J. Am. Chem. Soc.* **1986**, *108*, 2093.
(44) Bothner-By, A. A.; Stephens, R. L.; Lee, L.; Warren, C. D.; Jeanloz, R. W. *J. Am. Chem. Soc.* **1984**, *106*, 811.
(45) Bax, A.; Davis, D. G. *J. Magn. Reson.* **1985**, *63*, 207.
(46) Griesinger, C.; Ernst, R. R. *J. Magn. Reson.* **1987**, *75*, 261.
(47) Schmieder, P.; Zimmer, S.; Kessler, H. *Magn. Reson. Chem.* **1991**, *29*, 375.
(48) Mierke, D. F.; Kessler, H. *J. Am. Chem. Soc.* **1991**, *113*, 9466.
(49) Koning, T. M. G.; Boelens, R.; Kaptein, R. *J. Magn. Reson.* **1990**, *90*, 111.

while for the three methyl groups of the Boc group, the restraint was applied to the middle carbon atom with the addition of 200 pm to the upper restraint.

The ROE restrained molecular dynamics simulations were carried out with a time step of 1.0 fs for a duration of 100 ps. The atomic velocities were randomly applied following a Boltzmann distribution about the center of mass to obtain a temperature of 300 K. A skewed biharmonic constraining function was used for the ROE restraints; maximum forces of 5000 and 1000 kcal/mol pm were used for the lower and upper distance restraints, respectively. The use of a maximum force produces a linear function when there is a large difference between target and actual distances.

Three different MD simulations were carried out. The first two used the method of floating chirality for the glycine C^2H protons. The force constant for the angles C^2H–C^2–C^2H, C^2H–C^2–C^1, N–C^2–C^2H, and N–C^2–C^1 was set to zero within the force field parameter set. Simulations, as described above, were then run with the two different prochirality assignments. In the first simulation, the prochirality of the two protons quickly switched, while in the second the assignments were maintained. Using the prochirality assignments thus produced a simulation with the standard force field was carried out. There are only small differences in the three simulations, resulting from distorsions about the glycine. Therefore, only the results from this last simulation are presented (Table II).

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Supplementary Material Available: ^{13}C NMR spectra for compounds **5**–**23** (25 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.