21. A Simple and Effective Chemical Phosphorylation Procedure for Biomolecules

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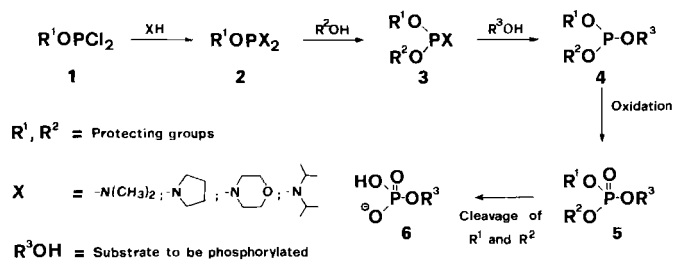
A general chemical phosphorylation method based on P(III) chemistry has been developed. The system is demonstrated for the phosphorylation of oligonucleotides, directly after their synthesis on a solid support, and for the *O*-phosphorylation of serine, threonine, and tyrosine as well as for a serine-containing peptide.

1. Introduction. – Many natural products exist as their phosphoric-acid esters. For their chemical synthesis, the phosphate moiety is mainly introduced *via* protected phosphoric-acid derivatives. The protecting groups should be stable during the phosphorylation procedure but should be cleaved selectively and quantitatively afterwards to yield the desired phosphorylated species. There exists a number of phosphate-protecting groups, but only a few of them seem to be of general applicability. We would like to report a very effective phosphorylation system which can be applied under very mild conditions and which is based on P(III) chemistry. It allows to use different protecting groups and, thus, to adapt them to the compound to be phosphorylated.

There are several reports on the synthesis of different alkoxybis(dialkylamino)-phosphines **2** [1–4]. They are easily prepared from PCl_3 *(Scheme 1)* which is reacted first with an alcohol to give the alkoxydichlorophosphine **1**. Further reaction of these compounds yields then the corresponding alkoxybis(dialkylamino)phosphines **2**. Compounds **2** are quite stable and easy to handle. They show a strong $p_\pi - d_\pi$ interaction of the N-atom of the dialkylamino functions with the P-atom [5]. This makes them easily susceptible for a protonation with very weak acids leading to an activated species which can react with another alcohol (R^2OH) to the dialkoxy(dialkylamino)phosphines **3**. The newly introduced alkoxy function reduces the $p_\pi - d_\pi$ interaction, and, therefore, compounds of type **3** are less susceptible for protonation (activation), and thus, compounds of type **3** behave like monofunctional reagents. Nevertheless, compounds of type **3** can still be activated under slightly more acidic conditions and allow their reaction with a

Scheme 1

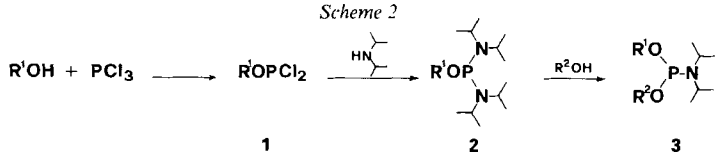


further alcohol or substrate bearing a suitable OH function (R^3OH) to yield a phosphite triester **4**. Oxidation of **4** leads to the corresponding phospho triesters **5**, and selective cleavage of R^1 and R^2 gives, finally, the phosphorylated compound **6**.

The intermediate dialkoxy(dialkylamino)phosphines **3** can be prepared very easily in high yields, and they are stable compounds, sometimes as stable as to allow their purification by silica-gel chromatography. Thus, they can be stored for a long time without decomposition in contrast to the corresponding dialkoxychlorophosphines sometimes used for phosphinylation [6]. Since the following steps (reaction of dialkoxy-(dialkylamino)phosphines with an alcohol and oxidation) proceed also in very high yields and under very mild conditions, this phosphorylation procedure should be of general use. The protecting groups can be adapted to the molecules to be phosphorylated.

2. Results and Discussion. – 2.1. *Phosphinylation Reagents.* To evaluate different phosphinylation reagents, we have synthesized the alkoxybis(dialkylamino)phosphines **2a** and **2b** and the dialkoxy(dialkylamino)phosphines **3a–c** *(Scheme 2)*. Thereby, we concentrated mainly on possibilities of phosphorylations of biomolecules like amino acids, peptides, nucleosides, and nucleotides (DNA, RNA). As dialkylamino function, we selected for all compounds **2** and **3** the $(i\text{-}Pr)_2N$ group. In the preparation of dichloro-(2-cyanoethoxy)phosphine (**1a**), we were not able to get a good yield of distilled product (22%) due to decomposition. Therefore, the crude mixture was reacted directly with $(i\text{-}Pr)_2NH$ which gave, after distillation, an overall yield of 57% of pure (2-cyano-ethoxy)bis(diisopropylamino)phosphine (**2a**). All the other intermediates and products could be obtained in reasonably good yields. Compounds **2a** and **b** were purified by distillation, whereas the actual phosphinylation reagents **3a–c** were so stable that they could be purified by short-column chromatography on silica gel [7].

Scheme 2

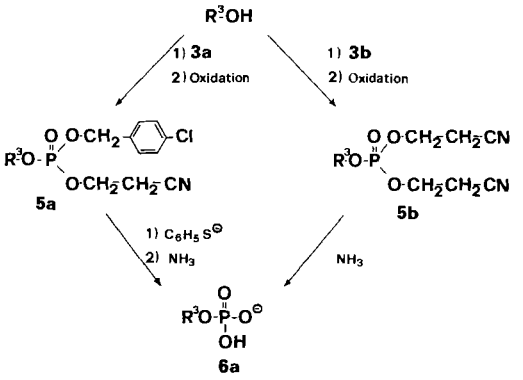


1 **2** **3**

	R^1	R^2	Yield [%]
1a	$NCCH_2CH_2$	–	89 (crude)
1b	$C_6H_5CH_2$	–	71
2a	$NCCH_2CH_2$	–	57
2b	$C_6H_5CH_2$	–	95
3a	$NCCH_2CH_2$	$ClC_6H_4CH_2$	94
3b	$NCCH_2CH_2$	$NCCH_2CH_2$	81
3c	$C_6H_5CH_2$	$C_6H_5CH_2$	78

Compounds **3a** and **b** were designed to be used mainly in connection with automated synthesis of DNA and RNA fragments on solid support. Most of the DNA fragments used in gene technology are used as their 5'-phosphates, and they are usually prepared by an enzymatic reaction using polynucleotide kinase and γ-^{32}P-ATP. A good chemical phosphorylation procedure could replace this enzymatic reaction and allow to work on a larger scale and with non-labelled DNA fragments where no radioactivity is needed (*e.g. Linker* fragments).

Scheme 3



5a, 5b R^3 = Protected DNA fragment attached to solid support
6a R^3 = Unprotected DNA fragment

In compound **3a**, we have combined the *p*-chlorobenzyl with the 2-cyanoethyl group. It can be applied to the phosphorylation of DNA fragments (R^3OH) whenever methoxy (dialkylamino)phosphoramidite building blocks were used for the synthesis of DNA on solid support [3] [8] *(Scheme 3)*. The unprotected phosphorylated DNA fragment **6a** is then obtained by the usual deprotection procedure. The *p*-chlorobenzyl group of **5a** is cleaved by nucleophilic attack with thiophenol which removes at the same time the methyl groups from the internucleotide phosphate linkages. The following NH_3 treatment, removing the protecting groups from the exocyclic amino function of the heterocyclic bases G, A, and C and cleaving the DNA from the support, is sufficient to remove also the 2-cyanoethyl group from the phospho diester yielding then the desired 5'-phosphorylated DNA fragments (see *Chapt. 2.2*).

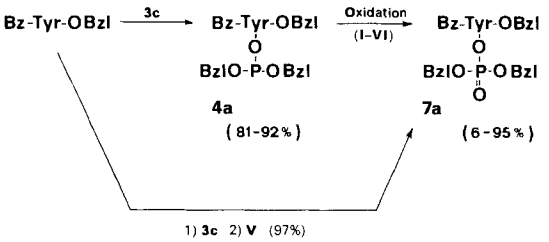
Compound **3b** was also designed to be used mainly in automated synthesis of phosphorylated DNA fragments, this time in combination with the more and more extensively applied (cyanoethyl)phosphoramidites [9–11]. After the synthesis of the phosphorylated DNA fragment on solid support **5b** *(Scheme 3)*, a single cleavage step with conc. NH_3 is sufficient to give directly the completely unprotected phosphorylated DNA fragment **6a** (see *Chapt. 2.2*). The cleavage of the first 2-cyanoethyl group from the phosphate moiety proceeds *via* a β-elimination, but during the cleavage of the second 2-cyanoethyl group, also an other mechanism seems to be involved [12].

The base-labile 2-cyanoethyl group can, of course, be replaced by other suitable protecting groups (*e.g.* $O_2NC_6H_4CH_2CH_2$ [13]; $CH_3SO_2CH_2CH_2$ [14]; $C_6H_5SO_2CH_2CH_2$ [15]; CCl_3CH_2 [6]; $NCCH_2C(CH_3)_2$ [16]). Furthermore, the system allows to be applied for phosphorylation of other substrates like sugars and glycerides and can also be used to link different molecules together *via* phospho-diester bridges.

Compound **3c** was synthesized to be used in phosphorylation of amino acids like serine, threonine or tyrosine at their OH group prior or in the course of peptide synthesis, or properly protected peptides itself. There, the system offers a good alternative to the

[1]) During the preparation of this manuscript, we realized that **3b** was also used by others for phosphorylation of DNA [11].

Scheme 4



Bz C_6H_5CO
Bzl $C_6H_5CH_2$

I	I_2/THF/lutidine/H_2O	6%
II	*tert*-Butyl hydroperoxide	51%
III	Iodobenzene diacetate	86%
IV	Tetrabutylammonium periodate	82%
V	*m*-Chloroperbenzoic acid	89%
VI	Peracetic acid	95%

phosphorylation by dibenzyl phosphoromonochloridate which has to be prepared *in situ* from dibenzyl phosphate and PCl_5 and which seems to be difficult to perform while giving rather poor yields [17–20]. The benzyl protecting groups are stable under the conditions used in peptide synthesis either in solution (*N*-Boc/benzyl-ester or *N*-Z/*tert*-butyl-ester strategy) or on solid support (Boc or Fmoc strategy). They can be removed quantitatively after synthesis by catalytic hydrogenation on Pd/C without side reaction, which cleaves off at the same time the *N*-Z and/or the benzyl-ester protection yielding directly the desired phosphorylated peptide. In order to investigate both reactions involved in the phosphorylation procedure (*Scheme 1*, phosphinylation and oxidation), we have isolated and purified the phosphite triester **4a**, after phosphinylation of *N*-benzoyltyrosine benzyl ester with **3c**, which we were able to obtain in a range of 81–92%, after short-column chromatography [7] (*Scheme 4*). For an evaluation of the oxidation step, **4a** was transformed to the corresponding phospho triester using different oxidation reagents (*Scheme 4*). Preliminary experiments had shown that the oxidation with I_2 in THF/lutidine/H_2O according to *Letsinger* and *Lunsford* [21] leads to side products when applied to intermediates of type **4** in solution (TLC). Performing this oxidation procedure with **4a** yielded **7a** in a yield of only 6%. Therefore, we were looking for alternative oxidation reagents like iodobenzene diacetate [22–24], tetrabutylammonium periodate [24] [25], *m*-chloroperbenzoic acid [26], peracetic acid, and *tert*-butyl hydroperoxide [27]. All of them gave good yields except *tert*-butyl hydroperoxide which yielded, after short-column chromatography, only 51% of **7a** together with 22% of *N*-benzoyltyrosine benzyl ester. The phosphorylation can, of course, also be carried out as a one-pot procedure; thus, in the phosphorylation of *N*-benzoyltyrosine benzyl ester using **3c** for the phosphinylation and *m*-chloroperbenzoic acid for the oxidation, we obtained 97% of **7a**.

2.2. *Phosphorylation of DNA Fragments on Solid Support.* To demonstrate the utility of our procedure, we phosphorylated the 18-mer DNA fragment d(TCCCATTCCC-GAGGAGCT) according to *Scheme 3*. The oligodeoxynucleotide was synthesized on controlled pore glass (CPG) as solid support using phosphoramidite chemistry [8] [28]

Table. *Cycles for Elongation* [33] *and Phosphorylation*

Reagent	Purpose	Elongation	Phosphorylation
3% $Cl_2CHCOOH$	detritylation	3 min	3 min
MeCN	wash	4 ×	4 ×
Activated amidites or phosphorylation mixture	condensation or phosphorylation	5 min	10 min
MeCN	wash	1 ×	1 ×
Ac_2O/Me_2NPy	capping	2 min	omitted
MeCN	wash	1 ×	1 ×
I_2 in THF/lutidine/H_2O	oxidation	2 min	2 min[a])
MeCN	wash	6 ×	6 ×

[a]) Occasionally occurring precipitate is removed by repeated washes with oxidation solution.

and our standard technology [29]. A *ca.* 15-fold excess of (2-cyanoethyl)amidite [9] [10] was applied in each elongation cycle.

The elongation cycle itself is outlined in the *Table*. After the complete synthesis of the DNA fragment, the material was splitted. One part of it was phosphorylated with **3a** and another part with **3b** by applying a standard elongation cycle with slightly longer reaction times *(Table)* and an excess of phosphinylation reagent. The resulting protected phosphorylated DNA fragments attached to the solid support, *i.e.* **5a** and **5b**, were then treated in a different manner in order to get the desired phosphorylated fragment **6a**. Compound **5a** was first reacted with thiophenol in order to remove the *p*-chlorobenzyl protecting group. Afterwards it was treated with conc. NH_3 at 56° to cleave all other protecting groups and to remove the fragment from the solid support. In the case of **5b**, a single treatment with conc. NH_3 at 56° was sufficient to give directly the desired phosphorylated DNA fragment **6a**. The crude material of both phosphorylations was subjected, together with the unphosphorylated DNA fragment as reference, to preparative polyacrylamide gel electrophoresis *(Fig. 1)*. Due to the additional negative charges in the phosphorylated species, it shows a greater mobility than the parent molecule and can, therefore, be separated easily. The good performance of the phosphorylation step can be judged by the fact that no unphosphorylated DNA fragment is left (*Fig. 1, lane 1* and *2*).

In order to check the phosphorylated DNA fragment, it was ligated to a 25-mer DNA fragment using a 30-mer as a template by DNA ligase.

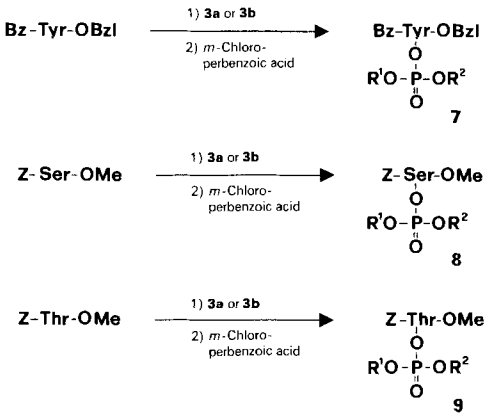
The resulting 43-mer was then labelled with ^{32}P and sequenced using a modified *Maxam-Gilbert* procedure which confirmed the right sequence [30] [31].

2.3. *Phosphorylation of Amino Acids and Peptides.* a) *Amino Acids.* The introduction of a phosphate group at the OH function of the amino acids serine, threonine, and tyrosine within a peptide may have a great influence on the activity and the physical behaviour of the parent peptides.

The chemical phosphorylation itself can be either performed on the level of a properly protected peptide having the site of phosphorylation still unprotected or by using properly protected phosphorylated amino acids in the actual peptide synthesis. The second way is certainly the preferred one if the peptide to be synthesized contains amino acids giving rise to side reactions during phosphorylation, *e.g.* tryptophan and methionine in the oxidation step of the described phosphorylation procedure.

Fig. 1. *UV-shadowing gel of the 5'-phosphorylated and unphosphorylated oligodeoxynucleotide d(TCCCATTCCCGAGGAGCT). Lane 1:* treatment of **5a** with thiophenol and NH_3; *lane 2:* treatment of **5b** only with NH_3; *lane 3:* unphosphorylated oligomer.

Scheme 5

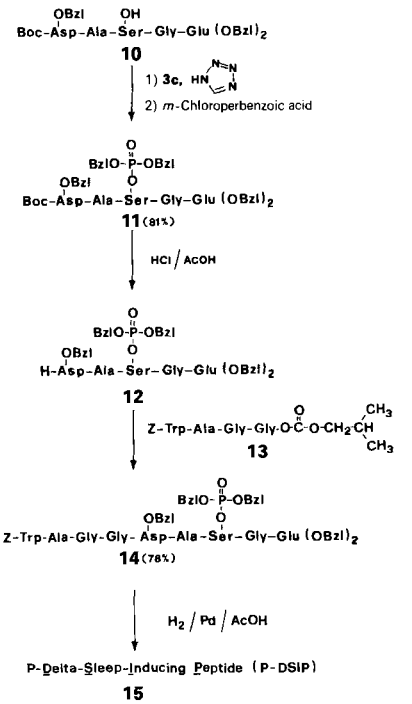


Bz C_6H_5CO
Bzl $C_6H_5CH_2$
Z $C_6H_5CH_2OCO$

	R^1	R^2	Yield [%]
7a	Bzl	Bzl	97
b	$ClC_6H_4CH_2$	$NCCH_2CH_2$	97
8a	Bzl	Bzl	98
b	$ClC_6H_4CH_2$	$NCCH_2CH_2$	93
9a	Bzl	Bzl	72
b	$ClC_6H_4CH_2$	$NCCH_2CH_2$	90

In order to evaluate both strategies, we have first phosphorylated protected serine, threonine, and tyrosine at their OH function according to *Scheme 5*. In the case of tyrosine, it was especially interesting to see how well this reaction can be used for the

Scheme 6



phosphorylation at phenolic OH functions. The phosphorylation using either reagent **3a** or **3c** was performed in a one-step fashion. The reaction proceeded in all cases in reasonably high yields and showed also a good yield when tyrosine was phosphorylated. The products were identified by spectroscopical methods.

b) *Phosphorylation of a Peptide.* As an example, we chose the protected pentapeptide Boc-Asp(OBzl)-Ala-Ser-Gly-Glu(OBzl)$_2$ (**10**) bearing a free OH function at the Ser residue. This pentapeptide is part of the delta-sleep-inducing peptide (DSIP), and its phosphorylated form P-DSIP is *ca.* 10 times more active than the unphosphorylated DSIP. The phosphorylation of **10** was performed in a one-pot mode using reagent **3c** for the phosphinylation step and *m*-chloroperbenzoic acid for the oxidation *(Scheme 6)*. The product **11** could be obtained after short-column chromatography in a yield of 81%. After cleavage of the Boc protecting group at the N-terminus, we obtained **12**, and this fragment was then condensed to the tetrapeptide **13** yielding the completely protected P-DSIP **14** in a yield of 78% *(Scheme 6)*[2]). Hydrogenation on Pd/C in AcOH gave directly the completely unprotected P-DSIP **15** which was purified by ion-exchange chromatography on *DEAE Sephadex*. *Fig. 2* shows an HPLC of the product after cleavage of the protecting groups and after purification by ion-exchange chromatography.

3. Conclusion. – The system described is very useful for a mild and high-yield phosphorylation of OH functions in properly protected amino acids, peptides, and DNA

[2]) Details of the synthesis of the building blocks will be reported elsewhere.

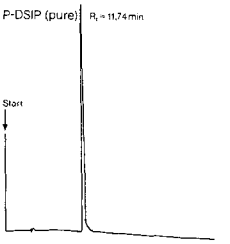


Fig. 2. *Reversed-phase HPLC of the purified phosphorylated Delta Sleep Inducing Peptide.* Conditions: *RP18,* 5–50% MeCN in 0.1% CF_3COOH/H_2O within 45 min, detection at 210 nm.

fragments. It can be extended to other substrates like sugars and glycerols *etc.* Work with other protecting groups is in progress.

Furthermore, by replacing one protecting group in compounds of type **3** by certain labels (fluorescing labels, spin labels), it should be possible to attach these in the same manner to biomolecules *via* phosphodiester bonds.

We would like to thank *P. Iaiza* for excellent technical assistance, *B. Hennequin* for the purification, *W. Lergier* for the HPLC and *M. Manneberg* for the quantitative determination of the amino-acid composition of the P-DSIP. Furthermore, we would like to thank our colleagues from the Central Research Units for C,H,N analysis (Dr. *A. Dirscherl*), ^{1}H-NMR spectra (Dr. *G. Englert*, Dr. *W. Arnold*), and MS (Dr. *W. Vetter, W. Meister*) and Miss *Bächler* for typing the manuscript.

Experimental Part

1. *General.* Pyridine: refluxed and distilled from TsCl, refluxed over CaH_2, distilled, and kept over molecular sieves (4 Å) in tightly closed bottles. CH_2Cl_2: dried over Na_2SO_4. MeCN: refluxed over CaH_2 and distilled just prior to use. Et_2O: dried over Na. Tetrazole *(Fluka)* was sublimed. Diisopropylammonium tetrazolide was prepared according to [3]. PCl_3, $C_6H_5CH_2OH$, *p*-$ClC_6H_4CH_2OH$, $NCCH_2CH_2OH$ and $(i\text{-}Pr)_2NH$ were all used without further purification. Protected amino acids were prepared according to [32]. More details about the synthesis of DNA fragments are given in [29] [33].

Short-column chromatography [7]: silica gel *60* (0.063–0.040 mm, *Merck*); solvent proportions in *v/v*. TLC: HPTLC-silica plates *(Merck)*; compounds containing P were visualized on TLC by *Zinzadze* spray [34] [35]. M.p.: not corrected. ^{1}H-NMR: at 80 and 250 MHz: chemical shifts in δ [ppm] rel. to TMS.

2. *Phosphines. Dichloro(2-cyanoethoxy)phosphine* (**1a**). To a soln. of PCl_3 (137.33 g, 85.5 ml, 1 mol) and dry pyridine (81 ml, 1 mol) in 200 ml of Et_2O under Ar at $-78°$ was added dropwise within 90 min 3-hydroxypropanenitrile (71.05 g, 68 ml, 1 mol). After removal of cooling, stirring was continued overnight at r.t. The precipitate was removed by filtration under Ar and washed twice with 100 ml of Et_2O. After evaporation of Et_2O, the oily residue was dried for 3 h at 14 Torr, thereby obtaining 153 g (89%) of crude **1a**. B.p. 95–100°/2 Torr. Since **1a** decomposed partly during distillation, it was used as such for the preparation of **2a**.

(Benzyloxy)dichlorophosphine (**1b**). To a soln. of PCl_3 (13.73 g, 8.55 ml, 0.1 mol) and dry pyridine (8.1 ml, 0.1 mol) in 100 ml of dry Et_2O under Ar was added benzyl alcohol (10.3 ml, 0.1 mol) in 50 ml of dry Et_2O within 90 min at $-78°$. The precipitate was removed by filtration under Ar and washed twice with 100 ml of Et_2O. After evaporation of Et_2O, the oily residue was bulb-to-bulb distilled, and 14.8 g (71%) of **1b** were obtained as a colourless oil which is not very stable. Therefore, it was used directly for the preparation of **2b**.

(2-Cyanoethoxy)bis(diisopropylamino)phosphine (**2a**). To a soln. **1a** of (37 g, 0.215 mol) in 300 ml of dry Et_2O under Ar and stirring, $(i\text{-}Pr)_2NH$ (268 ml, 1.9 mol) was added within 1 h at $-10°$. Stirring was continued overnight at r.t. The precipitated hydrochloride was removed by filtration under Ar and washed twice with 100 ml of Et_2O. The combined Et_2O solns. were evaporated, and the oily residue was distilled after addition of 0.5 g of CaH_2 over a 10-cm *Vigreux* column under vacuum, whereby we obtained 40.3 g (64%) of pure **2a**. B.p. 117°/0.5 Torr. Anal. calc. for $C_{15}H_{32}N_3OP$ (301.42): C 59.77, H 10.70, N 13.94; found: C 59.69, H 10.53, N 13.96.

Benzyloxybis(diisopropylamino)phosphine (**2b**) was prepared like **2a**, but starting from 70 mmol (14.6 g) of **1b** in 200 ml of Et_2O and 630 mmol (88 ml) of $(i\text{-}Pr)_2NH$. The crude **2b** (95%) obtained after evaporation of Et_2O was used directly for the preparation of **3c**.

(p-Chlorobenzyloxy)(2-cyanoethoxy)(diisopropylamino)phosphine (**3a**). To a soln. of 55 mmol (16.6 g) of **2a** and 25 mmol (4.30 g) of diisopropylammonium tetrazolide in 200 ml of CH_2Cl_2, 50 mmol (7.17 g) of *p*-chlorobenzyl alcohol were added under Ar and stirred. After complete addition, stirring was continued for another 2 h. The mixture was poured into 200 ml of sat. $NaHCO_3$ soln. which was then extracted 3 times with 100 ml of CH_2Cl_2. The combined org. layers were dried with Na_2SO_4 and evaporated. The oily residue was purified by short-column chromatography over 130 g of silica gel with CH_2Cl_2/AcOEt/Et_3N 80:15:5: 16.1 g (94%) of **3a** as colourless oil. ^{1}H-NMR ($CDCl_3$): 1.18, 1.21 (2*d*, $2(CH_3)_2CH$); 2.63 (*t*, OCH_2CH_2CN); 3.55–3.95 (2*m*, OCH_2CH_2CN, $2(CH_3)_2CH$); 4.55–4.80 (*m*, $C_6H_5CH_2$); 7.30 (br. *s*, C_6H_4). Anal. calc. for $C_{16}H_{24}ClN_2O_2$ (342.82): C 56.06, H 7.06, N 8.17; found: C 55.95, H 7.20, N 8.30.

Bis(2-cyanoethoxy)(diisopropylamino)phosphine (**3b**) was prepared like **3a**, but starting from 40 mmol (17 g) of **2a** and 20 mmol (3.44 g) of diisopropylammonium tetrazolide in 200 ml of CH_2Cl_2 and 44 mmol (3.0 ml) of 3-hydroxypropanenitrile. After short-column chromatography of the crude material over 60 g of silica gel with 700 ml of Et_2O/pentane 3:1, we obtained 8.8 g (81%) of pure **3b** as a colourless oil. ^{1}H-NMR ($CDCl_3$): 1.20 (*d*, $2(CH_3)_2CH$); 2.66 (*t*, $2OCH_2CH_2CN$); 3.58–3.68 (*m*, $2(CH_3)_2CH$); 3.78–3.94 (*m*, $2OCH_2CH_2CN$). Anal. calc. for $C_{12}H_{22}N_2O_2P$ (271.30): C 53.13, H 8.17, N 15.46; found: C 53.03, H 8.23, N 15.49.

Bis(benzyloxy)(diisopropylamino)phosphine (**3c**) was prepared in the same way as **3a**, but starting from 65 mmol (22 g) of **2b** and 30 mmol (5.2 g) of diisopropylammonium tetrazolide in 150 ml of CH_2Cl_2 and 60 mmol (6.2 ml) of benzyl alcohol. The resulting crude oily material was purified by short-column chromatography over 60 g of silica gel with CH_2Cl_2/AcOEt/Et_3N 80:15:5 and yielded 16 g (78%) of **3c** as a colourless oil. ^{1}H-NMR ($CDCl_3$): 1.23 (*d*, $2(CH_3)_2CH$); 3.45–4.05 (*m*, $2(CH_3)_2CH$); 4.81 (*d*, $2C_6H_5CH_2$); 6.90–7.20 (*m*, $2C_6H_5$). Anal. calc. for $C_{20}H_{28}NO_2P \cdot 0.1\ CH_2Cl_2$ (353.92): C 68.21, H 8.03, N 3.96; found: C 68.34, H 8.12, N 4.09.

3. *Phosphorylation of a DNA Fragment on Solid Support* via **5a** *or* **5b**. a) *Phosphinylation and Oxidation.* The DNA fragment d(TCCCATTCCCGAGGAGCT) was synthesized on controlled-pore glass as solid support using our standard procedure [29] [33]. The synthesis was started with 25 mg of functionalized support (0.67 mmol) and applying a soln. of 10 mg of the corresponding (cyanoethyl)phosphoramidite and 6 mg of tetrazole in 250 µl of anh. MeCN for each elongation cycle *(Table)*. The dimethoxytrityl protecting group at the 5'-end of this DNA fragment still attached in the completely protected form to the solid support was removed with 3% $Cl_2CHCOOH$ in dichloroethane [33]. Of this material, 10 mg were used for the phosphorylation with **3a** and 10 mg for the phosphorylation with **3b** using the phosphorylation cycle outlined in the *Table*. In each case we applied a soln. of 150 mg of **3a** or **3b** and 20 mg of tetrazole in 500 mg of anh. MeCN for the phosphorylation, and the oxidation was then performed with 0.2M I_2 in THF/lutidine/H_2O 2:1:2. The deprotection and purification was then performed according to *b*) or *c*).

b) *Deprotection of* **5a**. The support was treated with a mixture of thiophenol/dioxane/Et_3N 1:2:2 for 1.5 h. The mixture was filtered by suction into a soln. of I_2 in EtOH or 2.5% aq. NaOCl. The support was washed with dioxane, MeOH, THF, and Et_2O, dried and then transferred to an *Eppendorf* tube. After addition of 700 µl of conc. NH_3 (at least 25%), the tightly closed tube was kept overnight at 56°. After removal of the support, the NH_3 soln. was evaporated on a speed-vac concentrator. The residue was taken up in 300 µl of AcOH and precipitated by addition of 700 µl of Et_2O at r.t. After centrifuging, the pellet was dissolved in H_2O and part of this soln. was applied to gel electrophoresis on a 20% polyacrylamide gel. The bands of the product were visualized by UV shadowing *(Fig. 1)*, eluted with H_2O, and desalted on small reversed-phase columns. In this way, we obtained the pure phosphorylated DNA fragment **6a**.

For identification, the fragment was ligated with DNA ligase and ATP [36] to a 25-mer DNA fragment and using a 30-mer DNA fragment as template and yielding the expected 43-mer. Since the enzyme can only link 5'-phosphates of a DNA fragment to the 3'-end of another DNA fragment, this proved that the DNA fragment was really properly phosphorylated. The 43-mer DNA fragment obtained after ligation was then sequenced after labelling with γ-^{32}P-ATP and polynucleotide kinase [36] using a modified *Maxam-Gilbert* procedure [30] [31] which confirmed the right sequence (data not shown).

c) *Deprotection of* **5b**. This was done in the same way as for **5a**, except that in a single deprotection step, treatment with 700 µl of conc. NH_3 at 56° overnight gave directly the completely unprotected phosphorylated DNA fragment **6a** which was purified as in *b*) and identical with the product obtained in *b*).

4. *N-Benzoyl-O^4-[bis(benzyloxy)phosphino]-L-tyrosine Benzyl Ester* (Bz-Tyr(P(OBzl)$_2$)-OBzl; **4a**). To a soln. of 10 mmol (3.75 g) of Bz-Tyr-OBzl in 200 ml of anh. MeCN were added 20 mmol (1.40 g) of tetrazole and 11 mmol (3.80 g) of **3c** under Ar at r.t. and exclusion of moisture, whereupon a white precipitate was formed. Stirring was

continued for 1 h, and then the mixture was poured into 200 ml of sat. $NaHCO_3$ soln. After extraction with CH_2Cl_2 (3 × 150 ml), the org. layers were dried over Na_2SO_4 and evaporated: 7.7 g of an oil. This material was then purified by short-column chromatography over 50 g of silica gel. The column was eluted with the following solvent mixtures: 300 ml of Et_2O/pentane 2:3, 300 ml of Et_2O/pentane 1:1, and 300 ml of Et_2O/pentane 2:1. The pure fractions were collected (TLC: SiO_2, Et_2O, R_f 0.68) and evaporated. Crystallization of the residue from CH_2Cl_2/Et_2O/pentane afforded 5.4 g of **4a** as colourless needles. A second crop afforded another 0.3 g. Yield: 5.7 g (92%) of **4a**; m.p. 90–91°. In two other reactions starting from 3 mmol of Bz-Tyr-OBzl, the yield was 81%. ^{1}H-NMR ($CDCl_3$): 3.21 (*d*, *J* = 6, 2H–C(3)); 5.02 (*d*, *J* = 10, $(C_6H_5CH_2O)_2P$); 5.05–5.25 (*m*, $C_6H_5CH_2OO$, H–C(2)); 6.60 (br. *d*, NH); 6.95 (br. *s*, C_6H_4); 7.20–7.90 (*m*, $4C_6H_5$). Anal. calc. for $C_{37}H_{34}NO_6P$ (619.65): C 71.72, H 5.53, N 2.26; found: C 71.67, H 5.63, N 2.19.

5. *Oxidations of* **4a** *to* N-*Benzoyl*-O^4-*[bis(benzyloxy)phosphoryl]*-L-*tyrosine Benzyl Ester* (**7a**). a) *With* tert-*Butyl Hydroperoxide.* To a soln. of 1 mmol (620 mg) of **4a** in 50 ml of CH_2Cl_2, 2 mmol (0.29 ml) of *tert*-butyl hydroperoxide (70%; aq.) were added at r.t. and with stirring, whereupon the soln. turned yellow. Stirring was continued for 1 h when TLC showed complete disappearance of **4a** (silica gel, Et_2O, R_f 0.68). The mixture was poured into 50 ml of 10% $NaHSO_3$ soln. The org. layer was separated and the aq. layer extracted twice with 50 ml of CH_2Cl_2. The combined org. layers were dried with Na_2SO_4 and evaporated. The oily residue (650 mg) was purified by short-column chromatography on 10 g of silica gel using Et_2O/pentane 2:1. Two fractions were isolated in pure form. The first (450 mg; R_f 0.43, silica gel, Et_2O) yielded, after crystallization from Et_2O/pentane, 325 mg (51%) of pure **7a**. M.p. 84–85°. ^{1}H-NMR ($CDCl_3$): 2.74 (*d*, *J* = 6, 2H–C(3)); 5.05–5.30 (*m*, $(C_6H_5CH_2O_2P$, $C_6H_5CH_2OO$, H–C(2)); 6.66 (br. *d*, NH); 7.02 (br. *s*, C_6H_4); 7.30–7.90 (*m*, $4C_6H_5$). Anal. calc. for $C_{37}H_{34}NO_7P$ (635.65): C 69.91, H 5.39, N 2.20; found: C 69.69, H 5.36, N 2.11.

The 2nd fraction (110 mg; R_f 0.50; silica gel, Et_2O) yielded, after crystallization, 80 mg (22%) of Bz-Tyr-OBzl.

b) *With Iodobenzene Diacetate.* To a soln. of 1 mmol (620 mg) of **4a** in 50 ml of CH_2Cl_2, 2 mmol (640 mg) of iodobenzene diacetate were added at r.t. with stirring. Stirring was continued for 1 h, and then the mixture was poured into 50 ml of sat. $NaHCO_3$ soln. The org. layer was separated, and the aq. layer was extracted 4 times with 50 ml of CH_2Cl_2. The combined org. layers were collected and yielded, after evaporation, 1.1 g of an oil which was separated by short-column chromatography on silica gel (10 g) using 300 ml of pentane/Et_2O 1:2. The pure fractions were collected, and after evaporation and crystallization from CH_2Cl_2/Et_2O/pentane, 543 mg (86%) of pure **7a** were obtained, identical with a reference sample.

c) *With Tetrabutylammonium Periodate.* The reaction was carried out in the same way and with the same molar amounts as the oxidation with iodobenzene diacetate. TLC showed a complete reaction after 10 min. The mixture was evaporated and then purified by short-column chromatography over 10 g of silica gel using Et_2O. The pure fractions were collected and evaporated. Crystallization from pentane/CH_2Cl_2 yielded 520 mg (82%) of pure **7a**, identical with a reference sample.

d) *With* m-*Chloroperbenzoic Acid.* To a soln. of 0.5 mmol (310 mg) of **4a** in 20 ml of CH_2Cl_2, 1 mmol (172 mg) of *m*-chloroperbenzoic acid was added at r.t. with stirring. Stirring was continued for 2 h. After dilution with another 20 ml of CH_2Cl_2, it was washed twice with a 10% $NaHSO_3$ soln., then with H_2O and finally with a sat. $NaHCO_3$ soln. The org. layer was dried over Na_2SO_4 and evaporated: 310 mg of an oil. Crystallization from CH_2Cl_2/pentane yielded 280 mg (89%) of pure **7a**, identical with a reference sample.

e) *With Peracetic Acid.* To a soln. of 1 mmol (620 mg) of **4a** in 50 ml of CH_2Cl_2, 2 mmol (0.35 ml) of peracetic acid were added at r.t. with stirring. Stirring was continued for 1 h. The mixture was poured into 50 ml of sat. $NaHCO_3$. The org. layer was separated and the aq. layer extracted 4 times with 50 ml of CH_2Cl_2. The combined org. layers were dried with Na_2SO_4, and after evaporation, 650 mg of colourless **7a** were obtained. Crystallization from CH_2Cl_2/Et_2O/pentane yielded 605 mg (95%) of pure **7a**.

6. *One-pot Phosphorylations of Amino Acids.* N-*Benzoyl*-O^4-*[bis(benzyloxy)phosphoryl]*-L-*tyrosine Benzyl Ester* (**7a**). To a soln. of 1.2 mmol (448 mg) of Bz-Tyr-OBzl in 50 ml of anh. MeCN 2.4 mmol (170 mg) of tetrazole and 1.5 mmol (514 mg) of **3c** were added at r.t. with stirring. A few min after addition, a precipitate appeared. After 1 h, 1.7 mmol (290 mg) of *m*-chloroperbenzoic acid were added, whereupon the precipitate was dissolved. Stirring was continued for 1 h at r.t. Then, the mixture was taken up in 200 ml of Et_2O extracted 3 × with 5% aq. $NaHSO_3$ soln. (50 ml) and 3 × with sat. $NaHCO_3$ soln. (50 ml), the Et_2O soln. dried with Na_2SO_4, and evaporated. The resulting oil was dissolved in 10 ml of Et_2O, and pentane was added until the soln. became slightly cloudy and the product started to crystallize. After filtration, the crystals were washed with pentane and dried: 735 mg (97%) of **7a**, m.p. 86–87°, identical with **7a** obtained after oxidation of **4a**.

N-*Benzoyl*-O^4-*[(*p-*chlorobenzyloxy)(2-cyanoethoxy)phosphoryl]*-L-*tyrosine Benzyl Ester* (**7b**) was synthetized like **7a** by the one-pot procedure from 1.2 mmol (448 mg) of Bz-Tyr-OBzl and 1.5 mmol (515 mg) of **3a**. The crude product, after evaporation of the org. layer, could not be crystallized and was, therefore, separated by

short-column chromatography on 25 g of silica gel with 400 ml of CH_2Cl_2/MeOH 99:1 and then 400 ml of CH_2Cl_2/MeOH 98:2. The pure fractions yielded, after intensive drying of the residue, 725 mg (97%) of **7b** as an oil. ^{1}H-NMR ($CDCl_3$): 2.58–2.70 (*m*, OCH_2CH_2CN); 3.12–3.34 (2*dd*, 2H–C(3)); 4.14–4.30 (*m*, OCH_2CH_2CN); 5.05–5.30 (*m*, $ClC_6H_4CH_2O$, $C_6H_5CH_2O$, H–C(2)); 6.62–6.70 (2 br. *d*, NH); 6.94–7.05 (*m*, C_6H_4); 7.24–7.78 (*m*, $2C_6H_5$). Anal. calc. for $C_{33}H_{30}ClN_2O_7P \cdot 0.1\ CH_2Cl_2$ (641.53): C 61.97, H 4.75, N 4.57; found: C 61.51, H 4.97, N 4.41.

N-*Benzyloxycarbonyl*-O^3-*[bis(benzyloxy)phosphoryl]*-L-*serine Methyl Ester* (**8a**) was prepared like **7a** starting from 2 mmol (689 mg) of Z-Ser-OMe in 30 ml of anh. MeCN and 4 mmol (286 mg) of *m*-chloroperbenzoic acid. The resulting oil (1.3 g), after workup, was crystallized from 60 ml of pentane/Et_2O 5:1: 1.02 g (98%) of pure **8a**, m.p. 91–92°. ^{1}H-NMR ($CDCl_3$): 3.73 (*s*, CH_3O); 4.20–4.75 (*m*, H–C(2), 2H–C(3)); 5.05 (br. *d*, J = 8.8, 4H, $(C_6H_5CH_2O)_2P$); 5.15 (br. *s*, $C_6H_5CH_2OCO$); 5.60–5.90 (br. *d*, NH); 7.41 (br. *s*, $3C_6H_5$). Anal. calc. for $C_{26}H_{28}NO_8P$ (513.48): C 60.82, H 5.50, N 2.73; found: C 60.68, H 5.59, N 2.67.

N-*Benzyloxycarbonyl*-O^3-*[(p-chlorobenzyloxy)(2-cyanoethoxy)phosphoryl]*-L-*serine Methyl Ester* (**8b**) was prepared like **7b** starting from 4 mmol (1.09 g) of Z-Ser-OMe and 8 mmol (560 mg) of tetrazole in 100 ml of anh. MeCN to which were added 4.8 mmol (1.6 g) of **3a** and 6 mmol (10.4 g) of *m*-chloroperbenzoic acid. Workup as for **7b** afforded 2.7 g of an oil. A first short-column chromatography over 50 g of silica gel using 500 ml of CH_2Cl_2/MeOH 99:1, 500 ml of CH_2Cl_2/MeOH 98:2, and 500 ml of CH_2Cl_2/MeOH 57:3 yielded 1.42 g of pure **8b**, and a second one on silica using the same solvent gradient but 250 ml instead of 500 ml yielded another batch of 0.48 g of **8b**. Yield 1.90 g (93%). ^{1}H-NMR ($CDCl_3$): 2.59–2.70 (*m*, OCH_2CH_2CN); 3.76, 3.78 (2*s*, CH_3O); 4.05–4.63 (3 *m*, OCH_2CH_2CN, H–C(2), 2H–C(3)); 5.01, 5.05 (2*d*, $ClC_6H_4CH_2$); 5.12 (*s*, $C_6H_5CH_2$); 5.74, 5.82 (2*d*, NH); 7.25–7.48 (*m*, C_6H_5, C_6H_4). Anal. calc. for $C_{22}H_{24}ClN_2O_8P$ (510.87): C 51.72, H 4.74, N 5.48; found: C 51.54, H 4.91, N 5.51.

N-*Benzyloxycarbonyl*-O^3-*[bis(benzyloxy)phosphoryl]*-L-*threonine Methyl Ester* (**9a**) was prepared like **8a** starting from 2 mmol (535 mg) of Z-Thr-OMe in 50 ml of MeCN and 4 mmol (280 mg) of tetrazole, 2.4 mmol (827 mg) of **3c** and 2.5 mmol (430 mg) of *m*-chloroperbenzoic acid. The resulting oil (1.2 g) was purified by 3 short-column chromatographies on 20 g of silica gel each with CH_2Cl_2 and increasing amounts of MeOH (0.5–2%): total of 760 mg (72%) of pure **9a**. ^{1}H-NMR ($CDCl_3$): 1.36 (*d*, 3H–C(4)); 3.62 (*s*, CH_3O); 4.45 (*ddd*, H–C(2)); 4.95–5.05 (*m*, H–C(3), $(C_6H_5CH_2O)P$); 5.13 (*s*, $C_6H_5CH_2O$); 5.46 (*d*, J = 10, NH); 7.33 (br. *s*, $3C_6H_5$). Anal. calc. for $C_{27}H_{30}NO_8P$ (527.51): C 61.48, H 5.73, N 2.66; found: C 61.43, H 6.02, N 2.60.

N-*Benzyloxycarbonyl*-O^3-*[(p-chlorobenzyloxy)(2-cyanoethoxy)phosphoryl]*-L-*threonine Methyl Ester* (**9b**) was prepared like **9a** using 2.4 mmol (823 mg) of **3a**: 1.3 g of crude **9b** as an oil. Purification was performed by short-column chromatography over 40 g of silica gel with 300 ml of CH_2Cl_2/MeOH 99:1 and 300 ml of CH_2Cl_2/MeOH 98.5:1.5. The pure fractions (TLC) yielded 945 mg (90%) of **9b**. ^{1}H-NMR ($CDCl_3$): 1.38, 1.43 (2*d*, 3H–C(4)); 2.60–2.70 (2*t*, OCH_2CH_2CN); 3.70, 3.78 (2*s*, CH_3O); 4.05–4.15 (*m*, OCH_2CH_2CN); 4.44, 4.51 (*ddd*, H–C(2)); 4.99–5.10 (2*d*, 1*m*, $ClC_6H_4CH_2OP$ and H–C(3), resp.); 5.14 (*s*, $C_6H_5CH_2O$); 5.55 (*d*, NH); 7.30–7.45 (*m*, C_6H_4, C_6H_5). Anal. calc. for $C_{23}H_{26}ClN_2O_8P \cdot 0.1\ CH_2Cl_2$: C 52.02, H 4.95, N 5.25; found: C 51.87, H 5.17, N 5.35.

7. *Phosphorylation of Peptide* **10** *and its Transformation to P-DSIP* **15**. *Boc-Asp(OBzl)-Ala-Ser(PO(OBzl)$_2$)-Gly-Glu(OBzl)$_2$* (**11**). To a soln. of 0.5 mmol (424 mg) of **10** in 70 ml of anh. MeCN, 1 mmol (70 mg) of tetrazole and 0.6 mmol (207 mg) of **3c** were added at r.t. with stirring. Stirring was continued for 1 h, and then 0.8 mmol (140 mg) of *m*-chloroperbenzoic acid were added. After another h, the mixture was taken up in 100 ml of Et_2O and extracted 3 times with 10% aq. $NaHSO_3$ soln. (30 ml). The org. layer was dried with Na_2SO_4 and then evaporated: 700 mg of oil. This was separated by short-column chromatography over 30 g of silica gel using 200 ml of CH_2Cl_2, 200 ml of CH_2Cl_2/MeOH 99:1, 200 ml of CH_2Cl_2/MeOH 98:2, and 200 ml of CH_2Cl_2/MeOH 97:3. The pure fractions (TLC: silica gel $CHCl_3$/MeOH 19:1, R_f 0.44) gave 520 mg (94%) of **11** as an oil. This was precipitated from 100 ml of pentane/CH_2Cl_2 95:5. The precipitate was collected by filtration, washed with pentane, and dried: 450 mg (81%) of **11** as a colourless powder. Anal. calc. for $C_{57}H_{66}N_5O_{16}P$ (1108.15): C 61.78, H 6.00, N 6.32; found: C 61.61, H 6.09, N 6.50.

Asp(OBzl)-Ala-Ser(PO(OBzl)$_2$)-Gly-Glu(OBzl)$_2$ (**12**). Compound **11** (1 mmol, 1.10 g) was treated for 5 min at r.t. with 15 ml of 1N HCl/AcOH, and then the soln. was evaporated. The solid residue was taken up in 5 ml of DMF, adjusted with *N*-methylmorpholine to pH 8 and cooled to $-20°$.

Z-Trp-Ala-Gly-Gly-Asp(OBzl)-Ala-Ser(PO(OBzl)$_2$)-Gly-Glu(OBzl)$_2$ (**14**). The mixed anhydride **13** was prepared *in situ* from 1.1 mmol (576 mg) of Z-Trp-Ala-Gly-Gly-OH [31] in 5 ml of DMF by adding 1.1 mmol (0.121 ml) of *N*-methylmorpholine, followed by 1.1 mmol (0.143 ml) of isobutyl chloroformate at $-15°$. After an activation time of 2 min at $-15°$, the DMF soln. of **12** (see above) was added, and the soln. was kept for 10 min at $-15°$ and 1 h at r.t. It was poured into 100 ml of 5% aq. $NaHCO_3$ soln. and the resulting precipitate was collected by filtration, washed with H_2O, dried, and crystallized from MeOH/hexane: 1.15 g (69%) of **14**, m.p. 221°.

$[\alpha]_D^{20} = -14.6°$ ($c = 1$, DMF). Anal. calc. for $C_{78}H_{85}N_{10}O_{20}P$ (1513.56): C 61.90, H 5.66, N 9.25, P 2.05; found: C 61.70, H 5.79, N 9.40, P 2.08.

Trp-Ala-Gly-Gly-Asp-Ala-Ser(PO(OH)$_2$)-Gly-Glu (**15**). In 20 ml of AcOH/H_2O 4:1, 0.4 mmol (605 mg) of **14** were hydrogenated in the presence of 10% Pd/C. After complete uptake of H_2, the catalyst was removed by filtration, and the soln. was evaporated. The residue was lyophilized once from H_2O and then taken up in 5 ml of 0.2N AcOH and purified on a *DEAE-Sephadex* column (2.5 × 6 cm) using a linear gradient of 0.5N AcOH (300 ml) and 300 ml of 0.8N HCOOH as solvent [37]. The fractions containing the pure product were lyophilized: 144 mg of pure **15**. $[\alpha]_D^{20} = -19°$ ($c = 0.5$, 1N AcOH). Amino-acid ratio: Trp 0.95, Asp 1.00, Ser 0.70, Glu 0.96, Gly 2.90, Ala 1.99. Anal. calc. for $C_{35}H_{49}N_{10}O_{18}P \cdot 4H_2O$ (1000.86): C 42.00, H 5.74, N 13.99; found: C 42.26, H 5.69, N 14.03.

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