Pergamon

Tetrahedron Vol. 51, No. 25, pp. 6971-6976, 1995

0040-4020/95 $9.50+0.00

0040-4020(95)00344-4

ALLYL AS INTERNUCLEOTIDE PROTECTING GROUP IN DNA SYNTHESIS TO BE CLEAVED OFF BY AMMONIA

Frank Bergmann, Erich Kueng, Patrick Iaiza, and Willi Bannwarth*
Pharma Research Chemistry, F. Hoffmann-La Roche Ltd. CH-4002 Basel, Switzerland.

Abstract: Deprotection of allyl groups from internucleotide phosphotriester functions can be achieved not only with Pd^0-mediated cleavage in the presence of an appropriate nucleophile but also under standard conditions for DNA fragments using conc. ammonia at elevated temperature. This observation widens the scope for chemical phosphorylation procedures based on phosphoramidite chemistry and creates possibilities for the preparation of hybrid molecules composed of DNA and peptides without modification of the solid phase synthesis approach.

For the synthesis of DNA fragments on solid support the method of choice is the phosphoramidite approach developed by *Caruthers*.[1] The initial protecting group for the internucleotide linkage was methyl which had to be cleaved from the corresponding triesters by nucleophilic attack with thiophenol. Presently the 2-cyanoethyl group is favoured and its removal is achieved by β-elimination under mild basic conditions.[2]

The removal of allyl protecting groups under essentially neutral conditions mediated by Pd^0-catalysts has made this group attractive in synthetic organic chemistry and more recently in the peptide and nucleotide field.[3,4,5,6,7] In DNA synthesis, *Noyori* has also applied allyl and allyloxycarbonyl-protection for the internucleotide linkages and the exocyclic amino functions of cytosine, adenine and guanine, respectively.[8] Using this strategy, stepwise deprotection had to be performed to obtain the DNA fragments. Deprotection of the allyl groups with the Pd^0 catalyst and an appropriate nucleophile had to be carried out with the fragment still attached to the solid support to avoid contamination of the DNA fragment with the catalyst. This is then followed by basic treatment to release the fragment from the support material.

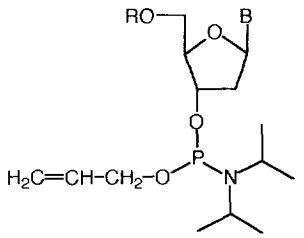
During our studies on phosphorylations of peptides and DNA using allyl-protection we experienced that conditions for the cleavage with Pd were difficult to optimize.[9,10] This holds especially true for the cleavage of allyl protecting groups on solid support.

One of our research interests is to synthesize chimeric molecules composed of peptides and DNA. We intended to combine the Fmoc strategy for peptide synthesis with DNA synthesis via the phosphoramidite approach with a minimum of deviation from standard protocols. The 2-cyanoethyl phosphoramidites cannot be applied in combination with the Fmoc strategy for peptide synthesis, since the deprotection of the Fmoc-group by 20% piperidine in DMF leads to a concomitant loss of the 2-cyanoethyl group from phosphotriesters. Alternatively, one can use a solution of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) for Fmoc-deprotections during solid phase peptide synthesis but these conditions also lead to a deprotection of the β-cyanoethyl group from phosphotriesters.[11]

Initial experiments on a protected dinucleoside monophosphate model revealed that the allyl group in phosphotriesters is completely stable under these conditions. However, we did observe partial cleavage of the allyl protecting group from phosphotriesters with ammonia solutions at r.t. . This prompted us to investigate whether we could optimize the ammonia cleavage conditions to

attain complete cleavage of the allyl groups of the phosphotriester internucleotide linkages of DNA fragments omitting Pd^0 catalysts.

To test this possibility we synthesized the allyl phosphoramidites **1a-e** from the corresponding 5'-DMTr- and the 5'-Fmoc-protected nucleoside by phosphinylation with allyloxy-bis-(diisopropylamino)phosphine in the presence of diisopropylammonium tetrazolide.[12] The phosphoramidites were purified by short column chromatography[13] and their identity verified by spectroscopic methods including ^{31}P-NMR (Fig.1).



	B	R	^{31}P (ppm)
1a	thymine	DMTr	148.3 ; 148.8
1b	N^6-benzoyladenine	DMTr	148.7 ; 148.8
1c	N^4-anisoylcytosine	DMTr	148.6 ; 149.2
1d	N^2-isobutyrylguanine	DMTr	148.3
1e	thymine	Fmoc	149.1 ; 149.4

Fig.1

In a first trial **1a** was employed for the synthesis of dT_9 (**2**) on CPG support functionalized with thymidine. As a control the identical sequence was prepared using β-cyanoethyl phosphoramidites. In addition, the same sequence was prepared by applying **1e** as building block. During this synthesis the Fmoc group was removed after each condensation with 2% DBU in DMF. The coupling yields of the three types of phosphoramidites were observed to be the same.

The oligonucleotide in which we applied 2-cyanoethyl phosphoramidites was deprotected under standard conditions with concentrated ammonia. In the case where we had applied allyl phosphoramidites different conditions were tested for the $Pd(PPh_3)_4$ mediated deprotection of the allyl internucleotide phosphotriesters as well as different temperatures for the cleavage with conc. ammonia according to Fig. 2.

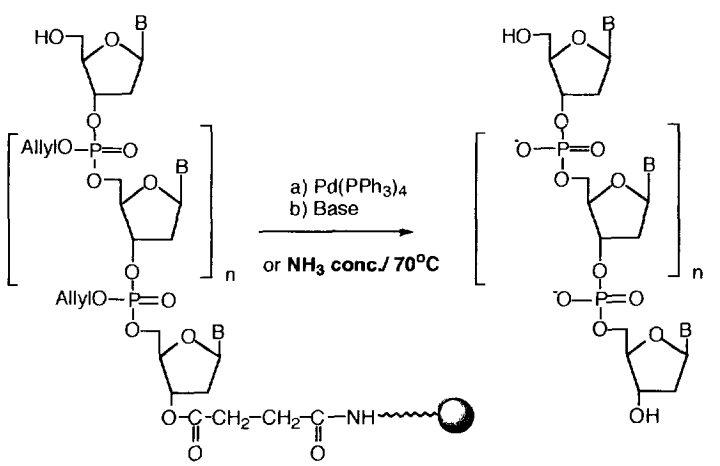
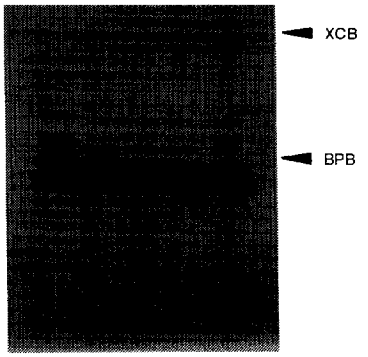


Fig.2

After deprotection the identity of the crude products from the different synthesis using the different phosphoramidites was investigated by polyacrylamide gel electrophoresis (PAGE) (Fig.3) and by reversed phase HPLC (data not shown).

Fig.3 PAGE of crude d(T_9) (**2**)



Lane 1 : Allyl phosphoramidites; Pd $(PPh_3)_4$ / morpholine (non optimized conditions); NH_3, rt.

Lane 2 : Allyl phosphoramidites; Pd $(PPh_3)_4$ / morpholine / THF / Dioxane / DMSO / 0.5 M HCl (optimized conditions); NH_3, rt.

Lane 3 : Allyl phosphoramidites; NH_3 conc., 70°C, 2h

Lane 4 : 2-Cyanoethyl phosphoramidites; NH_3 conc., 70°C, 2h (Comparison)

Lane 5 : Mix of lane 3 and lane 4 products

Cleavage by calf spleen phosphodiesterase followed by HPLC separation of the products yielded after complete degradation a ratio of dTp/dT of 8:1. These results clearly indicate that the product obtained from the cleavage by conc. ammonia for 2 hours at 70 °C leads to the same product as the Pd^0-mediated cleavage and the product of the synthesis performed with 2-cyanoethyl phosphoramidites. To show the generality of this observation we have synthesized the mixed sequence d(AAAACGACGGCCAGTG) (**3**) with 2-cyanoethyl- and allyl phosphoramidites, respectively. After ammonia deprotection the products of the two synthesis were compared by PAGE and reversed phase HPLC and found to be identical. The PAGE of the crude d(AAAACGACGGCCAGTG) (**3**) is shown in Fig.4. Cleavage with spleen phosphodiesterase resulted in a complete digestion to yield the monomers dAp, dCp, dGp, dTp and dG in the expected ratio.

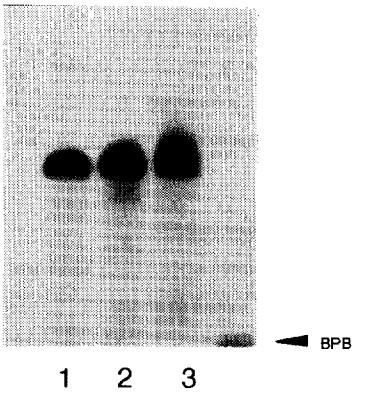


Fig.4 PAGE of crude $d(AAAACGACGGCCAGTG)_{3'}$ (**3**)

Lane 1 : 2-Cyanoethyl phosphoramidites; NH_3 conc., 70°C, 2h

Lane 2 : Allyl phosphoramidites; NH_3 conc., 70°C, 2h

Lane 3 : Allyl phosphoramidites;
a) $Pd^{(0)}$/ morpholine.
b) NH_3 conc., rt., 2h.

Neither in PAGE nor in HPLC we can observe smaller fragments from the ammonia cleavage of the oligonucleotides synthesized with allyl phosphoramidites. Thus, we surmise that this deprotection of the allyl protecting group from phosphotriesters does not proceed via an addition elimination process but rather a nucleophilic attack on the allyl group resulting in a shift of the double bond and release of the phosphodiester.

In summary, we were able to show that the allyl group of internucleotide linkages can not only be cleaved by Pd^0-catalysts in the presence of an appropriate nucleophile but also with conc. ammonia at elevated temperature which is identical with the standard conditions for deprotection of DNA fragments after solid phase synthesis.

Allyl groups on internucleotide phosphotriester functions are stable to 0.1 M DBU in DMF as shown on a dinucleoside monophosphate and in the synthesis where we applied the 5'-Fmoc derivative **1e**. These conditions can be used to remove Fmoc groups in peptide synthesis. Thus, one can synthesize hybrid molecules composed of peptides and DNA without modification of the solid phase approach. The synthesis of such molecules using this strategy will be reported in due course.

Furthermore, combinations between allyl and 2-cyanoethyl protection in chemical phosphorylation approaches based on phosphoramidite chemistry widen the scope of this approach.[14,15]

Combinations between allyl and 2-cyanoethyl phosphoramidites can also be used for the synthesis of DNA fragments. After removal of the 2-cyanoethyl group with 2% DBU the resulting phosphodiester function(s) can be manipulated and yet complete deprotection can be achieved afterwards with conc. ammonia.

Experimental

General: All solvents were of highest purity available. Tetrazole (*Fluka*) was sublimed. DBU, Pd $(PPh_3)_4$, *m*-chloroperbenzoic acid, dichloro acetic acid were from *Fluka*. Diisopropylammonium tetrazolide was prepared according to [12]. DBU stable sarcosine modified CPG support was synthesized according to.[16] (Allyloxy) bis (diisopropylamino) phosphine was prepared according to.[9] Short column chromatography (CC)[13] silicagel 60 (0.063-0.04 mm, *Merck*) TLC: HPTLC plates (*Merck*). HPLCs were run on a *LiChrosorb* RP 18 column 300 x 4 mm (*Merck*) at 40^o with a flow of 1 ml / min from 5 to 15% B in 20 min A: 0.1 M Triethylammonium acetate pH 7.0, B: MeCN. Polyacrylamid Gel Elektrophoresis (PAGE) was performed on 40 x 20 x 0.2 cm preparative Gels under denaturing conditions (7M urea) with 0.1 M Tris-borate EDTA pH 8.3 as buffer. Calf spleen phosphodiesterase was purchased from Boehringer Mannheim.

DNA synthesis employing the different phosphoramidites was technically performed as described earlier.[17] The dinucleoside monophosphate triester Tp(allyl)T for the initial experiments was synthesized from 5'-O-(4,4'-dimethoxytrityl) 3'-(allyloxy diisopropylamino phophinyl) thymidine (**1a**) and 3'-O-acetyl thymidine in analogy to Ref. 18 followed by cleavage of the acetyl group by NaOMe.

N^4- Anisoyl -5'-O-(4,4'-dimethoxytrityl) 3'-O-(allyloxy diisopropylamino phosphinyl) 2'-deoxy-cytidine (**1c**)

General procedure. A mixture of 20 mmol (13.3g) of N^4- anisoyl -5'-O-(4,4'-dimethoxytrityl) -2'-deoxy cytidine and 10 mmol (1.7 g) of diisopropylammonium tetrazolide was twice taken up in anh. MeCN and evaporated. To the residue in 200 ml of anh. CH_2Cl_2, 25 mmol (7.5 g) of allyloxy bis-(diisopropylamino) phosphine were added with stirring. After 10 min of stirring at r.t. the mixture was poured into 300 ml of sat. $NaHCO_3$ soln. and extracted with CH_2Cl_2 (3 x 200 ml). The combined org. layers were dried (Na_2SO_4) and evaporated. The crude compound was purified by CC (200 g

silicagel, CH_2Cl_2 / MeOH / Et_3N 94/4/2). The pure fractions (TLC CH_2Cl_2 / MeOH / Et_3N 94/4/2: Rf 0.28) evaporated and precipitated from CH_2Cl_2 (50 ml) into n-pentane (2 l) at -80° yielded 6.5 g (40%) of pure **1c** as diastereoisomers. ^{31}P-NMR ($CDCl_3$): 2 s: 148.6, 149.2 ppm. ^1H-NMR ($CDCl_3$): 1.06-1.18 (2 d, 2 $(CH_3)_2$ CH); 2.23-2.88 (2 m, H-C (2')); 3.36-3.70 (m, 2 H-C (5'), 2 $(CH_3)_2$ CH); 3.79-3.80 (2 s, CH_3O of DMTr); 3.68 (CH_3O of anisoyl); 3.96-4.30 (m, H-C (4'), CH_2-CH=CH_2); 4.54-4.75 (m, H-C (3')); 5.02-5.35 (m, CH_2=CH-CH_2); 5.73-6.03 (m, CH=CH_2); 6.30 (2 dd, H-C (1')); 6.85 (d, 4 arom. H of DMTr); 6.98 (d, 2 arom. of anisoyl); 7.16-7.47 (10 arom. H of DMTr; H-C (5)); 7.85 (2 arom. H of anisoyl); 8.30 (2 d, H-C (6)); 8.50 (br. s, NH).

*5'-O-(4,4'-Dimethoxytrityl) 3'-(allyloxy diisopropylamino phosphinyl) thymidine (**1a**)* was prepared in the same way. ^{31}P-NMR ($CDCl_3$): 2 s: 148.3, 148.8 ppm. ^1H-NMR ($CDCl_3$): 1.04-1.18 (2 d, 2 $(CH_3)_2$ CH); 1.40 (bs, CH_3); 2.23-2.63 (m, 2 H-C (2')); 3.28-3.70 (m, 2 H-C (5'), 2 $(CH_3)_2$ CH); 3.79 (s, 2 CH_3O); 3.94-4.06 (m, H-C (4')); 4.07-4.33 (m, CH_2-CH=CH_2); 4.60-4.73 (m, H-C (3')); 5.01-5.35 (m, CH_2=CH-CH_2); 5.70-6.02 (m, CH=CH_2); 6.40 (2 dd, H-C (1')); 6.83 (d, 4 arom. H of DMTr); 7.20-7.45 (m 9 arom. H of DMTr); 7.63 (d, H-C (6)); 8.25 (br. s, NH).

*N^6-Benzoyl-5,-O-(4,4'-dimethoxytrityl) 3'-(allyloxy diisopropylamino phosphinyl) -2'-deoxy adenosine (**1b**)* was prepared in the same way. ^{31}P-NMR ($CDCl_3$): 2 s: 148.7, 148.8 ppm. ^1H-NMR ($CDCl_3$): 1.11-1.28 (2 d, 2 $(CH_3)_2$ CH); 2.58-3.02 (m, 2 H-C (2')); 3.27-3.73 (m, 2 H-C (5'), 2 $(CH_3)_2$ CH); 3.77 (s, O CH_3); 4.02-4.43 (m, H-C (4'), CH_2-CH=CH_2); 4.70-4.85 (m, H-C (3')); 5.04-5.34 (m, CH_2=CH-CH_2); 5.75-6.04 (m, CH=CH_2); 6.52 (dd, H-C (1')); 6.76-8.03 (18 arom. H of DMTr and benzoyl);8.18-8.20 (2 s, H-C (8)); 8.74 (s, H-C (2)); 8.92 (br. s, NH).

*N^2-Isobutyryl-5'-O-(4,4'-dimethoxytrityl) 3'-(allyloxy diisopropylamino phosphinyl)-2'-deoxy guanosine (**1d**)* was prepared in the same way. ^{31}P-NMR ($CDCl_3$): 2 s: 148.3 ppm. ^1H-NMR ($CDCl_3$): 0.80-1.28 (m, 18 H, $(CH_3)_2$ CH); 1.78 (m, 1 H-C (2')); 2.52 (m, 1 H-C (2')); 2.94-3.20 (m, 2 CH $(CH_3)_2$, isobutyryl); 3.35-3.66 (m, 2 H-C (5'), 2 CH $(CH_3)_2$); 3.76-3.77 (2 s, O CH_3); 3.97-4.32 (m, H-C (4'), CH_2-CH=CH_2); 4.70-4.82 (m, H-C (3')); 5.02-5.43 (m, CH_2=CH-CH_2); 5.65-6.05 (m, CH=CH_2); 6.21 (dd, H-C (1')); 6.76-7.50 (13 arom. H of DMTr); 7.60 (br. s, NH); 7.77-7.78 (2 s, H-C (8)); 11.87 (br. s, NH).

5'-O-[(9H-Fluoren-9-yl) methoxycarbonyl] 3'-O-(allyloxy diisopropylamino phosphinyl) thymidine (**1e**). A mixture of 2 mmol (930 mg) of 5'-O-Fmoc thymidine and 1 mmol (170 mg) of diisopropylammonium tetrazolide was twice taken up in anh. MeCN and evaporated. To the residue in 50 ml of anh. CH_2Cl_2, 3 mmol (870 mg) of allyloxy bis-(diisopropylamino) phosphine were added with stirring. Reaction was continued for 4 h at r.t. The mixture was poured into 200 ml of 0.1 M triethylammonium acetate (pH 7.0) and extracted with CH_2Cl_2 (4x100 ml). The combined org. layers were dried (Na_2SO_4) and evaporated: 1.6 g of crude **1e** which was purified by CC (100 g silicagel, Et_2O, 400 ml). The pure fractions (TLC (Et_2O): Rf 0.46) coevaporated with MeCN (several times) gave 700 mg (54%) of pure **1e** as diastereoisomers. Anal. calc. for $C_{34}H_{42}N_3O_8P$ (651.7): C 62.66 H 6.50 N 6.45 found: C 62.21 H 6.58 N 6.26. ^{31}P-NMR ($CDCl_3$) : 149.4 , 149.1 ppm (2 s, 1:1 diastereoisomers).

dT_9 (**2**). *Synthesis with **1a***: The synthesis was started with 60 mg of CPG-support derivatized with thymidine (1.7 μmol). For each elongation 5 μmol (4 mg) of phosphoramidite **1a** and 100 μmol (6.6 mg) of tetrazole in 250 μl of anh. MeCN were applied. After the cleavage of the DMTr group after the last coupling, 30 mg of support was treated with 700 μl of conc. NH_3 at 70° C for 2 h in a tightly closed *Eppendorf* tube. After filtration the NH_3 soln. was evaporated on a *speed-vac* concentrator. The residue was taken up in 100 μl of water and centrifuged (15 min, 0 °C). From the supernatant the DNA was precipitated by the addtition of 200 μl of dioxane and 600 μl of THF. After centrifuging (15 min, 0 °C) the pellet was dissolved in water and analyzed by PAGE (Fig.3) and part of the material was purified by reversed phase HPLC. Alternatively, 3 mg (0.085 μmol) of the support material after the synthesis was treated under argon with $Pd(PPh_3)_4$ / morpholine in THF / dioxane / DMSO / 0.5 M HCl (2/2/2/1) at rt. The support was washed with

THF and acetone and treated afterwards with 500 µl of conc NH_3 at rt. for 2 h. After filtration the NH_3 soln. was evaporated, the residue dissolved in water and analyzed by PAGE (Fig.3) and by HPLC.

Synthesis with **1e**: The synthesis was started with 60 mg of CPG-support derivatized with sarcosine[16] and thymidine (3 µmol). After deprotection of the DMTr group for each elongation 30 µmol (20 mg) of **1e** and 100 µmol (17 mg) of tetrazole in 500 µl of anh. MeCN were applied (coupling time 6 min). The Fmoc deprotection after each coupling was performed with a 0.1 M DBU soln in MeCN (3 times for 1 min). After completion of the synthesis 7 mg of the support were deprotected with 600 µl of conc NH_3 in the way described above. Identity was checked by PAGE and HPLC comparison.

Synthesis with 2-cyanoethyl phosphoramidites: The synthesis was carried out on 60 mg CPG as solid support modified with thymidine (1.7 µmol) using the same excesses and techniques as described for **1a**. Deprotection with 600 µl of conc NH_3 was also done in the same way.

d (AAAACGACGGCCAGTG) (**3**): The synthesis was carried out applying the phosphoramidites **1a-1d** as well as the 2-cyanoethyl phophoramidites as described above for the synthesis of **2**.

Enzymatic digestion of DNA fragments with calf spleen phosphodiesterase :
Prior to digestion the fragments were purified by HPLC with a LiChrosorb RP 18 column (250 x 10 mm Merck) at 40°C with a flow rate of 4 ml/min, from 5% to 8% B in 10 min and from 8% to 20% B in 60 min (A= 0.1 M TEAA pH 7.0 , B= MeCN). 2 OD (2.7 nmol) of DNA in 20 µl of 12.5 mM sodium succinate/HCl pH 6.5 were treated with 20 milliunits of calf spleen phosphodiesterase at 37°C for 3h. After a 5 min denaturation at 95°C, the mixture was diluted with 100 µl H_2O, filtered and injected into the HPLC. The expected peak ratio of 1:8 for the dT and dTp was obtained for digestion of **2** and for the digestion of **3** a ratio of 6:4:1:4:1 for dAp:dCp:Tp:dGp:dG was obtained. No uncleaved oligonucleotide could be detected.

Acknowledgments: We would like to thank Dr. Christian Lehmann for the 5'-Fmoc-thymidine.

References and Notes

1. McBride, L. J.; Caruthers, M. H. *Tetrahedron Lett.* **1983,** *24*, 245.
2. Sinha, N.D.; Biernat, J.; McManus, J.; Koester, H. *Nucleic Acids Res.* **1984**, *12*, 4539.
3. Kunz, H.; Unverzagt, C.; *Angew. Chem. Int. Ed. Engl.* **1984**, *23*, 436.
4. Kunz, H.; Waldmann, H. *Angew. Chem. Int. Ed. Engl.* **1984**, *23*, 7.
5. Kunz, H.; Dombo, B. *Angew. Chem. Int. Ed. Engl.* **1988**, *27*, 711.
6. Hayakawa,Y.; Uchiyama, M.; Kato, H.; Noyori, R. *Tetrahedron Lett.* **1985**, *26*, 6505.
7. Hayakawa, Y.; Nobori, T.; Noyori, R.; Imai, J. *Tetrahedron Lett.* **1987**, *28*, 2623.
8. Hayakawa, Y.; Wakabayashi, S.; Kato, H.; Noyori, R. *J. Am. Chem. Soc.* **1990**, *112*, 1691.
9. Bannwarth, W.; Kueng. E. *Tetrahedron Lett.* **1989**, *30*, 4219.
10. Kitas, E. A.;Knorr, R.; Trzeciak, A.; Bannwarth, W. *Helv. Chim. Acta* **1991**, *74*, 1314.
11. Lehmann, C.; Xu, Y.-Z.; Christodoulou, C.; Tan, Z.-K.; Gait, M. J. *Nucleic Acids Res.* **1989**, *17*, 1994.
12. Barone, A. D.; Tang, J. Y.; Caruthers, M. H. *Nucleic Acids Res.* **1984**, *12*, 4051.
13. Hunt, B. J.; Rigby, W. *Chem.Ind. (London)* **1967**, 1868.
14. Bannwarth, W.; Trzeciak, A. *Helv. Chim. Acta* **1987**, *70*, 175.
15. Pirrung, M. C.; Shuey, S. W. *J. Org. Chem.* **1994**, *59*, 3890.
16. Brown, T.; Pritchard, L.E.; Turner, G.; Salisburg, S.A. *J. Chem. Soc., Chem. Commun.* **1989**, *89*, 175.
17. Bannwarth, W. *Chimia* **1987**, *41*, 302.
18. Ji, Y.; Bannwarth, W.; Luu, B. *Tetrahedron* **1990**, *46*, 487.

(*Received in Germany* 10 *April* 1995; *accepted* 4 *May* 1995)