Benzyloxy(diisopropylamino)methylphosphine: A Powerful Reagent for the Synthesis of Methylphosphonopeptides

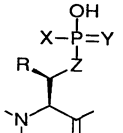
Jac C.H.M. Wijkmans, Wendelien Ottenheim, Esther Kuyl-Yeheskiely, Gijs A. van der Marel, Wim Bloemhoff, Jacques H. van Boom*

Leiden Institute of Chemistry, Gorlaeus Laboratories, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands

Received 19 July 1994

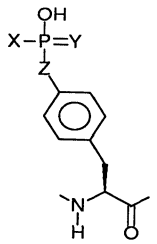
The preparation of methylphosphonylated amino acids and peptides using *O,O*-bis(benzotriazol-1-yl) methylphosphonate and the favourable novel reagent benzyloxy(diisopropylamino)methylphosphine [benzyl (*N,N*-diisopropyl)methylphosphonamidite] is described.

Protein phosphorylation mediated by protein kinases and phosphatases is now generally recognized as one of the major mechanisms by which eukaryotic cells regulate various cellular processes.[1] The biological relevance of these intriguing processes has urged many groups to synthesize modified phosphoamino acids and peptides. As a consequence, several modifications of the natural monophosphate function in serine, threonine and tyrosine (compounds **1a–3a**) have been published, i.e. phosphorothioate **1b–3b**,[2,3] phosphonate **1c–3c**,[4] hydroxyphosphonate **3d**,[5] fluorophosphonate **3e**[5] and difluorophosphonate **3f**.[5,6] As part of an ongoing program directed toward the preparation of phosphoamino acids, phosphopeptides and analogs thereof,[2,7] we now report the first synthesis of methylphosphonylated derivatives **1d** and **3g** using the established reagent *O,O*-bis(benzotriazol-1-yl) methylphosphonate **(4)** and the new methylphosphinylating reagent benzyloxy(diisopropylamino)-methylphosphine [benzyl (*N,N*-diisopropyl)methylphosphonamidite, **10**].



a: X = OH, Y = Z = O
b: X = OH, Y = S, Z = O
c: X = OH, Y = O, Z = CH_2
d: X = Me, Y = Z = O

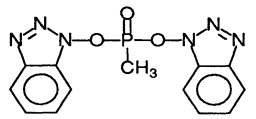
1 R = H
2 R = Me



a: X = OH, Y = Z = O
b: X = OH, Y = S, Z = O
c: X = OH, Y = O, Z = CH_2
d: X = OH, Y = O, Z = CH(OH)
e: X = OH, Y = O, Z = CHF
f: X = OH, Y = O, Z = CF_2
g: X = Me, Y = Z = O

3

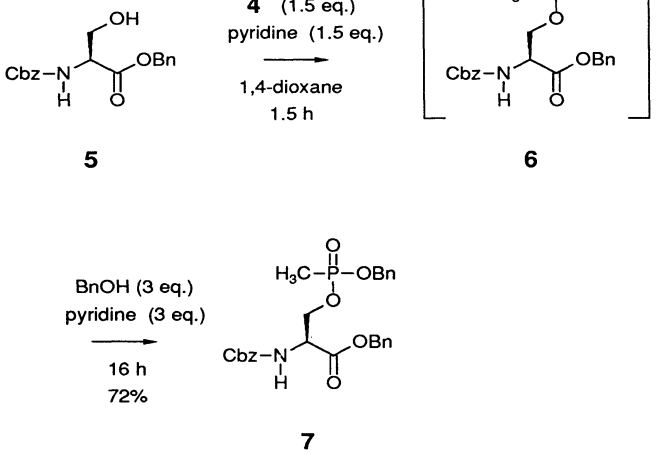
Originally, the methylphosphonate moiety was introduced to mimic the 3′ → 5′-internucleotide phosphate diester linkage in DNA.[8] In an earlier paper from this laboratory, it was disclosed that readily available *O,O*-bis(benzotriazol-1-yl) methylphosphonate[9] **(4)** is a convenient reagent for the synthesis of methylphosphonate-modified DNA fragments. In addition, the hydroxybenzotriazole approach also proved to be satisfactory in the synthesis of D-*myo*-inositol methylphosphonolipids and methylphosphonates.[10] On the basis of this information, the bifunctional reagent **4** should give access to methylphosphonylated amino acids and peptides.



4

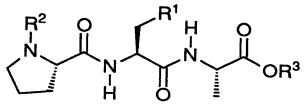
Indeed, reaction of the properly protected serine derivative **5** (Scheme 1) with a slight excess of phosphonylating reagent **4** showed complete disappearance of starting material in under 2 hours, as gauged by TLC analysis. The in situ formed benzotriazolyl ester **6** reacted slowly (16 hours) with benzyl alcohol to yield, after purification, the diastereomeric methylphosphonate diester **7** (see Table). The versatility of the two-step hydroxybenzoriazole approach was further demonstrated by the phosphonylation of the serinyl moiety in tripeptide **8a**. Thus, reaction of the primary hydroxy group of the tripeptide with **4** and subsequent addition of benzyl alcohol gave methylphosphonopeptide **8b** in a good yield.

Despite the successful synthesis of tripeptide **8b** via post-assembly phosphonylation of **8a** in solution, the use of **4** is not completely satisfactory for the solid-phase assembly of phosphonopeptides due to its high susceptibility to hydrolysis and prolonged reaction times. A pos-

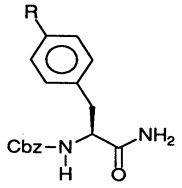


5 **6** **7**

Scheme 1 Bt = benzotriazol-1-yl



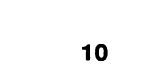
8 a: R^1 = OH, R^2 = Cbz, R^3 = Bn
b: R^1 = OPO_2BnCH_3, R^2 = Cbz, R^3 = Bn
c: R^1 = OPO_2HCH_3, R^2, R^3 = H



12 a: R = OH
b: R = OPO_2BnCH_3

sible alternative reagent for the methylphosphonylation of peptides on a solid support is bis(diisopropylamino)methylphosphine **(9)**, which has been previously used for the introduction of 3′ → 5′-internucleotide methylphosphonate diester linkages in DNA.[11] However, the general applicability of methylphosphine **9** is limited due to the occurence of side reactions, e.g. transesterification.[12] In order to circumvent this problem, **9** was converted into the monofunctional reagent benzyloxy(diisopropylamino)methylphosphine [benzyl (*N,N*-diisopropyl)methylphosphonamidite, **10**] by treatment with an excess of benzyl alcohol under the agency of catalytic 2,4,6-trimethylpyridine hydrochloride. Purification of the crude product by silica gel chromatography gave homogeneous **10** which could be stored for months without degradation.

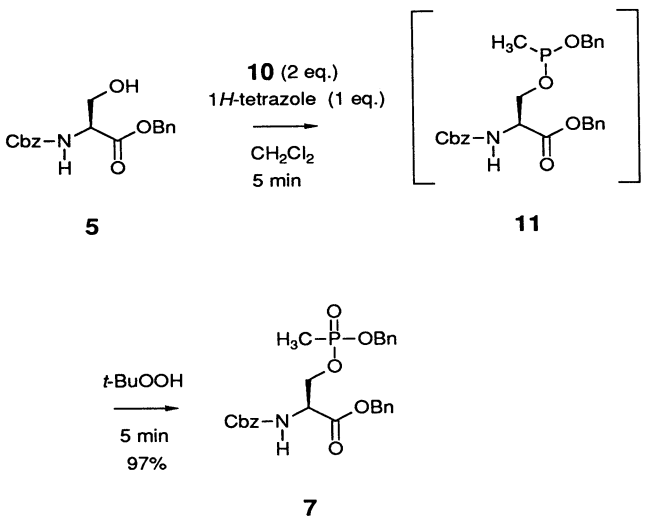
9



10

The favourable properties of the new monofunctional reagent **10** were demonstrated by the preparation of serine methylphosphonate **7**. To this end, serine derivative **5** was allowed to react with excess methylphosphonamidite **10** in the presence of an equimolar amount of 1*H*-tetrazole (Scheme 2). Monitoring of the reaction by ^{31}P NMR revealed rapid conversion (i.e. 5 min) of **5** into the intermediate phosphonite **11** (δ_P 186.7 and 185.1). The in situ oxidation of **11** with *tert*-butyl hydroperoxide went to completion within 5 min to give, after workup and column chromatography, methylphosphonate **7** as a mixture of two diastereoisomers in an excellent yield. The spectroscopic data of **7** were in every aspect identical with those of the same derivative obtained via the hydroxybenzotriazole method. In a similar fashion, phosphinylation of the phenolic hydroxy function of tyrosine derivative **12a** with **10** occurred smoothly to give, after oxidation, methylphosphonotyrosine derivative **12b**.

The efficacy of reagent **10** was illustrated further in the post-assembly or global phosphonylation of tripeptide **8a**. Thus, treatment of the tripeptide with excess **10** in the presence of 1*H*-tetrazole gave, after oxidation, the



5 **11** **7**

Scheme 2

fully protected methylphosphonylated peptide **8b** in a high yield. Ensuing hydrogenolysis of the benzyloxycarbonyl and benzyl protective groups from **8b** was easily effected over 10% palladium on activated charcoal to furnish the completely deblocked methylphosphonate monoester **8c** in a quantitative yield.

In conclusion, the results presented in this paper demonstrate that the new trivalent reagent benzyl (*N,N*-diisopropyl)methylphosphonamidite **(10)** is a highly efficient tool for the preparation of a new class of phosphopeptide analogs. Solid-phase synthesis of methylphosphonopeptides using amidite **10** is at present under investigation.

CH_2Cl_2 was distilled from CaH_2 and stored over molecular sieves (4Å). 1,4-Dioxane was freshly distilled prior to use from $LiAlH_4$. CH_3CN (Rathburn HPLC-grade) was stored over molecular sieves (4Å). Triethylammonium bicarbonate (TEAB, 2 M) buffer was prepared by passing a stream of CO_2 gas through a mixture of triethylamine (825 mL) and H_2O (2175 mL) at 0°C until at pH 7. TEA and 1*H*-tetrazole were purchased from Janssen. *tert*-Butyl hydroperoxide (80% solution in di-*tert*-butyl peroxide) was purchased from Merck-Schuchardt. $PhCH_2OCO$–Ser–OCH_2Ph and $PhCH_2OCO$–Tyr–NH_2 were obtained from NovaBiochem. Tripeptide **8a** was synthesized by classical methods.[13] All amino acids have the L-configuration. Reactions were carried out at ambient temperature unless noted otherwise. TLC analysis was performed on Schleicher and Schüll DC Fertigfolien F1500 LS254. Compounds were visualized by UV (254 nm) and TDM (*N,N,N′,N′*-tetramethyl-4,4′-diaminodiphenylmethane) reagent.[14] Column chromatography was performed on Kieselgel 60, 230–400 mesh (Merck). Mass spectra were obtained with a Finnigan MAT SSQ 710 (Finnigan MAT, San José) spectrometer equipped with an electrospray interface. ^1H, ^{13}C and ^{31}P NMR spectra were recorded on a Jeol JNM-FX 200 spectrometer, operating at 200, 50.1 and 80.7 MHz, respectively. The ^{13}C spectra were monitored using the Attached Proton Test (APT) technique. 2D (^1H–^1H COSY, ^1H–^{13}C COSY, ^1H–^{31}P COSY) NMR spectra were recorded at 300 MHz on a Bruker WM-300 spectrometer interfaced with an ASPECT 2000 computer. Chemical shifts (δ) are given in ppm relative to the signal for internal Me_4Si for ^1H, and to the signal for internal $CHCl_3$ (δ 77.0) or MeOH (δ 49.0) for ^{13}C. Sodium 4,4-dimethyl-4-silapentanesulfonate was used as internal reference for samples in D_2O. ^{31}P chemical shifts are given to 85% H_3PO_4 as external standard.

Table. Relevant Data of Methylphosphonylated Compounds **7**, **8b** and **12b**

Product	Yield[a,b] (%)	MS (*m/z*) $[M+H]^+$	^{31}P NMR[c] δ	^1H NMR[d] δ, *J* (Hz)	^{13}C NMR[e] δ, *J* (Hz)
7	72, A 97, B	498	33.0, 32.9	7.4–7.2 (m, 15H, H_{arom}), 5.2–4.9 (m, 6H, $PhCH_2$), 4.6 (m, 1H, Hα), 4.4–4.3 (m, 2H, Hβ), 1.39, 1.37 (2d, 3H, PCH_3, $^2J_{H,P}$ = 17.6)	168.9 (C=O Ser), 155.8 (C=O Cbz), 136.0, 135.9, 135.8, 134.9 (C_q of Ph), 128.5–127.8 (CH_{arom}), 67.4, 67.3, 67.2, 67.0 ($PhCH_2$), 65.1 (d, Cβ Ser, $^2J_{Cβ,P}$ = 5.9), 54.5 (d, Cα Ser, $^3J_{Cα,P}$ = 5.9), 10.9 (d, PCH_3, $^1J_{C,P}$ = 143.5)
8b	75, A 86, B	666	34.8, 34.4	7.4–7.2 (m, 15H, H_{arom}), 5.1–5.0 (m, 6H, $PhCH_2$), 4.7–4.6 (m, 1H, Hα Ser), 4.5–4.4 (m, 1H, Hα Ala), 4.3–4.2 (m, 2H, Hβ Ser), 4.1–4.0 (m, 1H, Hα Pro), 3.6–3.5, 3.5–3.4 (2m, each 1H, Hδ Pro), 2.2–2.1, 2.0–1.8 (2m, 1H and 3H, resp., Hβ Pro, Hγ Pro), 1.6–1.3 (m, 6H, Hβ Ala, PCH_3)	172.2, 172.1, 171.9, 168.2 (C=O Ala, Pro, Ser), 156.0 (C=O Cbz), 136.3–135.5 (C_q of Ph), 128.6– 127.5 (CH_{arom}), 68.0, 67.9, 67.1, 66.8, 65.5, 65.4, 64.9 (Cβ Ser, $PhCH_2$), 61.3 (Cα Pro), 53.5 (Cα Ser), 48.3 (Cα Ala), 47.0 (Cδ Pro), 29.9 (Cγ Pro), 24.4 (Cβ Pro), 17.5 (Cβ Ala), 10.8, 10.7 (2d, PCH_3, $^1J_{C,P}$ = 143.5 and 142.0, resp.)
12b	94, B	483	28.8	7.4–7.0 (m, 14H, H_{arom}), 6.54, 6.13 (2bs, each 1H, NH_2), 5.98 (d, 1H, NH, $J_{HαNH}$ = 5.7, 5.2–5.0 (m, 4H, $PhCH_2$), 4.5–4.4 (m, 1H, Hα), 3.1–2.9 (m, 2H, Hβ), 1.58, 1.57 (2d, 3H, PCH_3, $^2J_{H,P}$ = 17.6)	173.5 (C=O Tyr), 155.9 (C=O Cbz), 149.1 (d, Cζ, $^2J_{Cζ,P}$ = 7.3), 136.0 (C_q of Z), 135.7 (d, C_q of OBn, $^3J_{C,P}$ = 7.3), 133.4 (Cγ), 130.6–127.8 (Cδ, CH_{arom}), 120.5 (d, Cε, $^3J_{Cε,P}$ = 4.4, 67.7 (d, CH_2 of OBn, $^2J_{C,P}$ = 5.9), 66.9 (CH_2 of Cbz), 55.6 (Cα), 37.6 (Cβ), 11.5 (d, PCH_3, $^1J_{C,P}$ = 145.0)

[a] Isolated yields.
[b] A: hydroxybenzotriazole method; B: methylphosphonamidite method.
[c] Spectra recorded in $CDCl_3$ for **7** and **12b**, and in CD_3OD for **8b**.
[d] Spectra recorded in CD_3OD for **7** and **8b**, and in $CDCl_3$ for **12b**.
[e] Spectra recorded in $CDCl_3$.

Benzyloxy(diisopropylamino)methylphosphine [Benzyl (*N,N*-Diisopropyl)-methylphosphonamidite, 10]:
Benzyl alcohol (0.72 mL, 7.0 mmol) and 2,4,6-trimethylpyridine hydrochloride[12] (80 mg, 0.5 mmol) were dried by coevaporation with CH_3CN (3 × 5 mL) and dissolved in CH_2Cl_2 (5 mL). **9** (1.23 g, 5.0 mmol) was then added. After 24 h, triethylamine (TEA) (0.5 mL) was added and the mixture was concentrated. Purification of the resulting residue by column chromatography [light petroleum (bp 40–60 °C)/TEA, 19:1 v/v; R_f 1.00] yielded **10** (1.20 g, 95%) as a colourless liquid.

^1H NMR ($CDCl_3$): δ = 7.3–7.2 (H_{arom}), 4.7–4.6 (m, 2H, CH_2 of OBn), 3.6–3.5 (m, 2H, CH of *i*-Pr), 1.27 (d, 3H, PCH_3, $^2J_{H,P}$ 8.2), 1.21, 1.12 (2 d, each 6H, CH_3 of *i*-Pr, ^3J 6.9 and 6.7, resp.).

^{13}C NMR ($CDCl_3$): δ = 140.0 (d, C_q of OBn, $^3J_{C,P}$ 7.3), 128.1, 127.0, 126.9 (CH_{arom}), 68.4 (d, CH_2 of OBn, $^2J_{C,P}$ 19.1), 44.1 (d, CH *i*-Pr, $^2J_{C,P}$ 8.8), 24.7, 24.0 (2 d, CH_3 of *i*-Pr, $^3J_{C,P}$ 8.8 and 7.3, resp.), 17.9 (d, PCH_3, $^1J_{C,P}$ 11.7).

^{31}P NMR ($CDCl_3$): δ = 122.9.

Synthesis of Methylphosphonylated Amino Acids and Peptides; General Procedure:

Method A (Hydroxybenzotriazole Procedure):
To a stirred solution of **5** (165 mg, 0.50 mmol) or **8a** (250 mg, 0.50 mmol), dried by repeated coevaporation with dioxane (3 × 5 mL), in dioxane (2 mL) were added pyridine (60 μL, 0.75 mmol) and a solution of *O,O*-bis(benzotriazol-1-yl) methylphosphonate (**4**) in dioxane (0.2 M, 3.75 mL, 0.75 mmol). After 1.5–2 h, TLC analysis showed the absence of starting compound. Benzyl alcohol (155 μL, 1.5 mmol) and pyridine (120 μL, 1.5 mmol) were added, and the reaction mixture was stirred for 16 h. The mixture was then diluted with CH_2Cl_2 (100 mL), washed with TEAB (1 M, 3 × 30 mL), dried ($MgSO_4$) and concentrated. Purification of the residue by column chromatography furnished the corresponding methylphosphonylated compounds **7** and **8b**.

Method B (Methylphosphonamidite Procedure):
A mixture of the appropriate compound (0.50 mmol) and phosphinylating reagent **10** (1.5–2 eq.) was dried by evaporation with CH_3CN (3 × 10 mL) and dissolved in CH_3CN (5 mL) or CH_2Cl_2 (5 mL). 1*H*-Tetrazole (35 mg, 0.50 mmol), dried by evaporation with CH_3CN (3 × 5 mL), in dry CH_3CN (1 mL) was added. When ^{31}P NMR and TLC analysis[15] showed complete conversion of the starting compound into the respective phosphonite, the mixture was treated with *t*-BuOOH (0.5 mL) for 5 min. Extractive workup as described above followed by column chromatography gave **7**, **8b**, and **12b** as an oil.

Deprotection of Methylphosphonate 8b:
A solution of compound **8b** (207 mg, 0.31 mmol) in H_2O/MeOH (5% v/v, 5 mL) was hydrogenated in the presence of 10% palladium on charcoal (100 mg) for 24 h. The catalyst was removed by filtration, and the filtrate concentrated and lyophilized from H_2O to obtain homogeneous **8c** (109 mg, 100%) as a white solid.

MS (*m/z*): 352 $[M+H]^+$

^1H NMR (D_2O): δ = 4.60 (t, 1H, Hα Ser, $J_{α,β}$ 4.8), 4.45 (dd, 1H, Hα Pro $J_{α,β}$ 6.6 and 8.6), 4.28 (q, 1H, Hα Ala, $J_{α,β}$ 7.1), 4.1 (m, 2H, Hβ Ser), 3.5–3.3 (m, 2H, Hδ Pro), 2.5–2.4 (m, 1H, $Hβ^a$ Pro), 2.2–2.0 (m, 3H, $Hβ^b$ Pro, Hγ Pro), 1.38 (d, 3H, Hβ Ala, $J_{α,β}$ 7.2), 1.27 (d, 3H, PCH_3, $^2J_{H,P}$ 16.5).

^{13}C NMR (D_2O): δ = 177.0, 170.7, 170.4 (C=O Ala, Pro, Ser), 63.5 (Cβ Ser), 60.3 (Cα Pro), 55.2 (d, Cα Ser, $^3J_{C,P}$ 5.9), 49.7 (Cα Ala), 47.1 (Cδ Pro), 30.3 (Cγ Pro), 24.3 (Cβ Pro), 17.1 (Cβ Ala), 11.6 (d, PCH_3, $^1J_{C,P}$ 136.3).

^{31}P NMR (D_2O): δ = 28.3.

(1) Krebs, E.G.; Beavo, J.A. *Ann. Rev. Biochem.* **1979**, *48*, 923. Cohen, P. *Nature* **1982**, *296*, 613.
(2) **1b, 2b**: De Bont, H.B.A.; Moree, W.J.; Van Boom, J.H.; Liskamp, R.M.J. *J. Org. Chem.* **1993**, *58*, 1309.

(3) **3b**: Kitas, E.; Küng, E.; Bannwarth, W. *Int. J. Pept. Protein Res.* **1994**, *43*, 146.
(4) **1c**: Minowa, N.; Hirayama, M.; Fukatsu, S. *Tetrahedron Lett.* **1984**, *25*, 1147.
Schöllkopf, U.; Busse, U.; Lonsky, R.; Hinrichs, R. *Liebigs Ann. Chem.* **1986**, 2150.
Valerio, R.M.; Alewood, P.F.; Johns, R.B. *Synthesis* **1988**, 786.
Tong, G.; Perich, J.W.; Johns, R.B. *Tetrahedron Lett.* **1990**, *31*, 3759.
Jiao, X.-Y.; Chen, W.-Y.; Hu, B.-F. *Synth. Commun.* **1992**, *22*, 1179.
Shapiro, G.; Buechler, D.; Ojea, V.; Pombo-Villar, E.; Ruiz, M.; Weber, H.-P. *Tetrahedron Lett.* **1993**, *34*, 6255.
Hamilton, R.; Shute, R.E.; Travers, J.; Walker, B.; Walker, B.J. *ibid.* **1994**, *35*, 3597.
2c: Ruiz, M.; Ojea, V.; Shapiro, G.; Weber, H.-P.; Pombo-Villar, E. *ibid.* **1994**, *35*, 4551.
3c: Bayle-Lacoste, M.; Moulines, J.; Collignon, N.; Boumekouez, A.; De Tinguy-Moreaud, E.; Neuzil, E. *Tetrahedron* **1990**, *46*, 7793.
Cushman, M.; Lee, E.-S. *Tetrahedron Lett.* **1992**, *33*, 1193.
Garbay-Jaureguiberry, C.; McCort-Tranchepain, I.; Barbe, B.; Ficheux, D.; Roques, B.P. *Tetrahedron Asymmetry* **1992**, *3*, 637.
(5) Burke, T.R. Jr.; Smyth, M.S.; Nomizu, M.; Otaka, A.; Roller, P.P. *J. Org. Chem.* **1993**, *58*, 1336.
(6) Wrobel, J.; Dietrich, A. *Tetrahedron Lett.* **1993**, *34*, 3543.
Burke, T.R. Jr.; Smyth, M.S.; Otaka, A.; Roller, P.P. *ibid.* **1993**, *34*, 4125.
Smyth, M.S.; Burke, T.R. Jr. *ibid.* **1994**, *35*, 551.
(7) De Bont, H.B.A.; Veeneman, G.H.; Van Boom, J.H.; Liskamp, R.M.J. *Recl. Trav. Chim. Pays-Bas* **1987**, *106*, 641.
Kuyl-Yeheskiely, E.; Tromp, C.M.; Geluk, A.; Van der Marel, G.A.; Van Boom, J.H. *ibid.* **1988**, *107*, 567.
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.
Dreef-Tromp, C.M.; Erkelens, C.; Van der Marel, G.A.; Van Boom, J.H. *J. Am. Chem. Soc.* **1989**, *111*, 6470.
Van Oijen, A.H.; Erkelens, C.; Van Boom, J.H.; Liskamp, R.M.J. *ibid.* **1989**, *111*, 9103.
De Bont, H.B.A.; Van Bom, J.H.; Liskamp, R.M.J. *Recl. Trav. Chim. Pays-Bas* **1990**, *109*, 27.
De Bont, H.B.A.; Van Boom, J.H.; Liskamp, R.M.J. *Tetrahedron Lett.* **1990**, *31*, 2497.
Dreef-Tromp, C.M.; Lefeber, A.W.M.; Van der Marel, G.A.; Van Boom, J.H. *Synthesis* **1992**, 1269.
Moree, W.J.; Van der Marel, G.A.; Van Boom, J.H.; Liskamp, R.M.J. *Tetrahedron* **1993**, *49*, 11055.
Van Oijen, A.H.; Huck, N.P.M.; Kruijtzer, J.A.W.; Erkelens, C.; Van Boom, J.H.; Liskamp, R.M.J. *J. Org. Chem.* **1994**, *59*, 2399.
(8) Miller, P.S.; Agris, C.H.; Blake, K.R.; Murakami, A.; Spitz, S.A.; Reddy, M.P.; Ts'O, P.O.P. In *Nucleic Acids: The Vectors of Life*; Pullman, B.; Jortner, J., Eds.; D. Reidel Publishing Co.: Dordrecht, 1983.
(9) Marugg, J.E.; De Vroom, E.; Dreef, C.E.; Tromp, C.M.; Van der Marel, G.A.; Van Boom, J.H. *Nucleic Acids Res.* **1986**, *14*, 2171.
(10) Dreef, C.E.; Douwes, M.; Elie, C.J.; Van der Marel, G.A.; Van Boom, J.H. *Synthesis* **1991**, 443 and references cited therein.
(11) Roelen, H.C.P.F.; Van den Elst, H.; Dreef, C.E.; Van der Marel, G.A.; Van Boom, J.H. *Tetrahedron Lett.* **1992**, *33*, 2357.
(12) Jäger, A.; Engels, J. *Tetrahedron Lett.* **1984**, *25*, 1437.
(13) Bodanszky, M.; Bodanszky, A. *The Practice of Peptide Synthesis*; Springer-Verlag: Berlin, 1984.
(14) Von Arx, E.; Faupel, M.; Brugger, J. *J. Chromatogr.* **1976**, *120*, 224.
(15) TLC analysis [light petroleum (bp 40–60 °C)/ethyl acetate, 1 : 1 v/v] was performed using a small sample (0.1 mL) taken from the reaction mixture and oxidized with *t*-BuOOH (50 μL).