Characterization of Synthetic Parathyroid Hormone Analogues and of Synthetic Byproducts

Lynn Caporale,*,† Ruth Nutt,† Jay Levy,† Jack Smith,‡ Byron Arison,‡ Carl Bennett,† Georg Albers-Schonberg,‡ Steven Pitzenberger,† Michael Rosenblatt,† and Ralph Hirschmann‡,§

Merck Sharp & Dohme Research Laboratories, West Point, Pennsylvania 19486, and Rahway, New Jersey 07065

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A combination of analytical techniques including NMR spectroscopy, FAB mass spectrometry, UV spectroscopy, and tryptic mapping has allowed us to detect and characterize byproducts due to incomplete deprotection and premature chain termination formed during a synthesis of the 32 amino acid peptide hormone analogue [Nle^{8,18},Tyr^{34}]bPTH(3-34)amide. Our ability to characterize these byproducts has enabled us to modify the synthesis, resulting in a significant improvement in yield. NMR spectroscopy has also proven to be the method of choice for the detection of the oxidation of methionine.

Introduction

The need for a battery of independent methods for structure confirmation and assessment of purity of synthetic peptides is well recognized. There exists an equally important need to characterize byproducts in order to guide future syntheses and to facilitate yield improvement. Analytical techniques routinely used in nonpeptidal organic chemistry are now increasingly able to provide valuable information about the structure and purity of molecules of the complexity of peptide hormones. In a program involving the syntheses of closely related analogues of peptides of medium and large size, methodology that simplifies the characterization of such analogues[1] is desirable. We report here the use of a variety of analytical methods to characterize byproducts arising during the synthesis of parathyroid hormone analogues. We demonstrate that such characterization can be used to guide the improvement of the synthesis in order to eliminate the formation of byproducts.

Parathyroid hormone is an 84 amino acid peptide that plays a central role in regulating blood calcium levels.[2] An analogue missing 50 residues at the carboxyl end, [Nle^{8,18},Tyr^{34}]bPTH(1-34)NH$_2$ (Figure 1), is equipotent on a molar basis with intact parathyroid hormone in essentially all available assays. Parathyroid hormone is highly sensitive to truncation at its amino end. For example, [Nle^{8,18},Tyr^{34}]bPTH(3-34)NH$_2$ is an antagonist in most (but not all) assays.[2]

This report focuses on the use of several analytical approaches, including spectroscopy and peptide mapping, to identify major byproducts in the synthesis of the 3-34 analogue and to improve the yield.

Results and Discussion

[Nle^{8,18},Tyr^{34}]bPTH(3-34)amide (I) was synthesized by the solid-phase method of Merrifield[3] on an ABI instrument on *p*-methyl benzhydrylamine resin. Protecting groups were cleaved, and the peptide removed from the resin by the use of a modification of the low/high HF procedure of Tam and Merrifield.[4] Purification was achieved by gel filtration and HPLC.

The synthesis of I afforded a nearly equal amount of a byproduct II, eluting prior to the desired material on reversed-phase HPLC (Figure 2). Amino acid analyses following hydrolysis with 6 N HCl for 22 h did not distinguish between I and II, but UV spectroscopy indicated that II, but not I, contained formyl tryptophan.[5] This was confirmed by NMR spectroscopy, which revealed formyl protons at 9.10 and 9.45 ppm in II, absent in I (Figure 3). These two protons could be shown to represent formyl rotamers since heating to 70 °C caused the two peaks to coalesce at 9.23 ppm in a temperature-related reversible manner. Similarly, the C-7 proton of Trp was seen in II at 7.84 and 8.27 ppm, coalescing on heating at 8.0 ppm. It was concluded that II represents a byproduct, which retained the N^{in}-formyl blocking group on tryptophan due to incomplete deprotection in our hands under the conditions of the S_N2/S_N1 HF cleavage procedure described by Tam.[4] The FAB mass spectrum of II synthesized independently by S_N1 HF deblocking gave a molecular ion consistent with the proposed structure [Nle^{8,18},Trp(For)^{23},Tyr^{34}]bPTH(3-34)NH$_2$ (calcd 3946.62, found 3946.28). Treatment of II with hydrazine afforded I (Figure 2). Characterization of byproduct II allowed us almost to double the yield of the desired product by preparative conversion of II → I with hydrazine. In a subsequent experiment, use of the modified Tam procedure, in which the ratio of thiocresol to cresol was increased, avoided the formation of II altogether.

The synthesis of I afforded two additional byproducts, III and IV, which size-exclusion chromatography indicated to be of smaller molecular weight than I. The major byproduct, III, was purified to homogeneity via HPLC. The amino acid analysis suggested truncation at Glu^{19}. Truncation was confirmed by a comparison of tryptic maps of I and the synthetic peptide [Nle^{8,18},Tyr^{34}]bPTH(1-34)-amide (V) (M + H$^+$ calcd 4088.84, found 4088.71) with that of III. A tryptic digest of V (Figure 4) afforded after HPLC separation the four cleavage products expected from its sequence, each of which had the correct amino acid composition. Peptide mapping of a tryptic digest of I gave, as anticipated, the three fragments common with IV and a unique fourth fragment with the predicted amino acid analysis. A tryptic digest of compound III was consistent with its being a truncated byproduct, as it gave only the C terminal peptide common to compounds I and V and

* To whom inquiries should be directed.
† West Point, PA.
‡ Rahway, NJ.
§ Present address: Department of Chemistry, University of Pennsylvania, Philadelphia, PA 19104.

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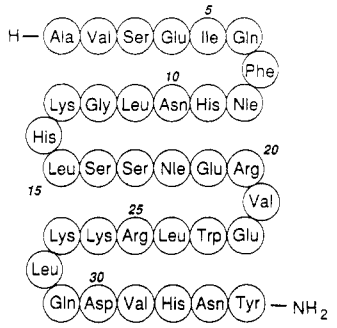


Figure 1. Sequence of parathyroid hormone analogue [Nle^{8,18},Tyr^{34}]bPTH(1–34)NH$_2$.

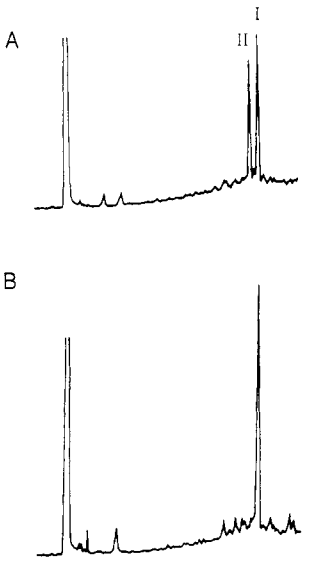


Figure 2. (A) Reverse-phase HPLC of I, synthesized by standard methodology, revealing major byproduct II. (B) Treatment of the above mixture with hydrazine converted II to I; Vydac C_4, 5 μm, 20% B to 35% A over 30 min. A = 5% CH_3CN containing 0.1% TFA; B = 0.1% TFA in CH_3CN.

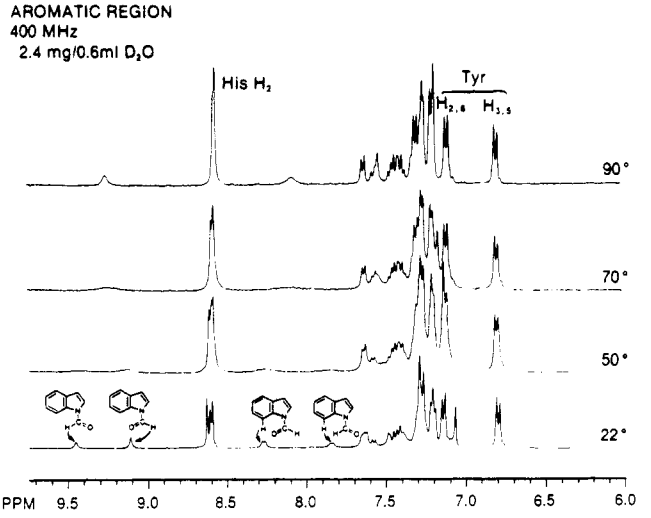
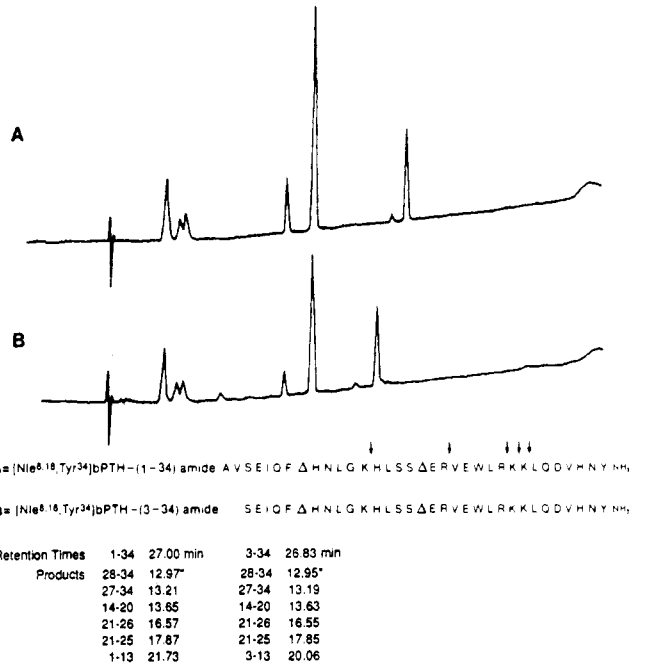


Figure 3. NMR spectrum of aromatic region of byproduct II, demonstrating retention of formyl protecting group on tryptophan. Signals from the Trp rotamers coalesce reversibly as the temperature is raised.

an additional peptide (Figure 5).

Thus, tryptic mapping can be useful in permitting the rapid identification of those regions of large molecules in which byproduct formation has occurred. Tryptic mapping should be particularly useful in an analogue program in-



Retention Times	1-34	27.00 min	3-34	26.83 min
Products	28-34	12.97*	28-34	12.95*
	27-34	13.21	27-34	13.19
	14-20	13.65	14-20	13.63
	21-26	16.57	21-26	16.55
	21-25	17.87	21-25	17.85
	1-13	21.73	3-13	20.06

Figure 4. Tryptic digests of 1–34 and 3–34 analogues, demonstrating the shifted position of the amino-terminal tryptic peptide. Amino acid residues are indicated by the standard single-letter code in which R = arginine and K = lysine. Δ is used here to indicate norleucine.

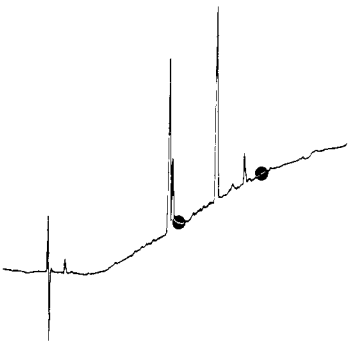


Figure 5. Tryptic digest of major termination byproduct III. The positions of missing tryptic fragments 3–13 and 14–20 are indicated by dark circles on the baseline.

volving the syntheses of closely related compounds. (For example, we have recently used tryptic mapping to characterize synthetic hPTH (1–84) (submitted).)

Sequence analysis of the truncated byproduct III indicated no free α-amino group. The possibility of trifluoroacetylation or pyro Glu formation were considered as likely causes of the observed premature chain termination at Glu[19] in III. However, fluorine NMR spectroscopy revealed the absence of trifluoroacetyl, thus eliminating the former as a possible mechanism of termination. The molecular ion observed upon FAB mass spectroscopy was not only clearly inconsistent with pyro Glu formation, but it also strongly suggested acetylation at Glu[19] as the mechanism of chain termination. (M + H$^+$ found 2155.94, calcd pyro Glu 2092.0, acetyl Glu 2155.46.) Indeed, the NMR spectrum of III confirmed the presence of an acetyl at 2.04 ppm. Acetic acid was subsequently found as a contaminant in the purchased specimen of Boc-Nle used to form the symmetric anhydride for the addition of Boc-Nle[18]. Concomitant formation of acetic anhydride was the most likely source for the production of III. Acetic acid presumably was generated by slow hydrolysis of ethyl acetate, which had been used by the manufacturer in the

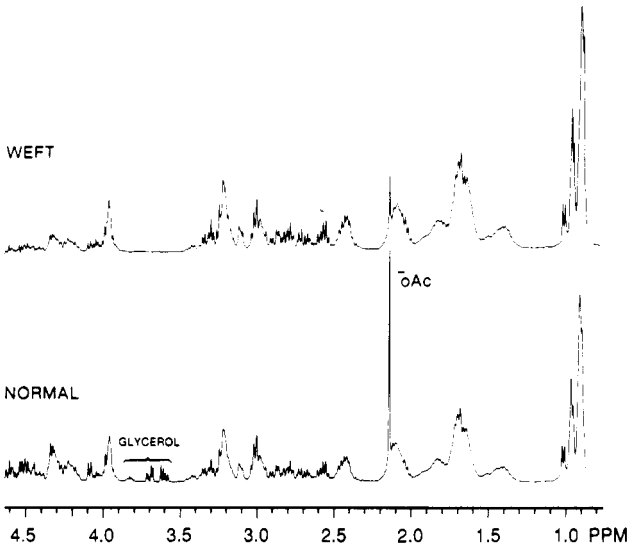


Figure 6. WEFT NMR spectrum indicates that unexpected signal is due to a low molecular weight contaminant.

isolation of commercially available Boc-Nle. Rotary evaporation of a solution of Boc-Nle in carbon tetrachloride enabled us to remove the contaminant ethyl acetate.

Having been able to characterize III to be Ac[Tyr^{34}]-bPTH(19–34)amide avoided our engaging in pointless endeavors to minimize pyro Glu formation or trifluoroacetylation, but instead identified a straightforward way to improve future yields.

FAB mass spectroscopy, NMR spectroscopy, and amino acid analysis revealed the minor truncated component IV to be Ac[Tyr^{34}]bPTH(24–34)amide (acetyl at 2.02 ppm, M + H$^+$ calcd 1455.70, found 1455.91). This product presumably was also truncated by acetic acid contamination observed in the Boc-Trp(For) used during incorporation of residue 23.

III and IV accounted for approximately 13% of the product of this synthesis. This termination byproduct peak was avoided in syntheses of PTH analogues prepared following characterization of III and IV.

An additional concern in our syntheses of PTH analogues has been a good analytic method to detect oxidation of methionine. Amino acid analysis or Edman degradation are not definitive methods for detecting methionine sulfoxide, as these analytical procedures can alter the oxidation state of methionine. In our hands, NMR spectroscopy has been the method of choice for the identification and quantitation of methionine sulfoxide in peptides. Oxidation has a significant effect on the methionine methyl signal. For example, in a synthesis of the PTH analogue [Tyr^{34}]bPTH(7–34)amide, with unoxidized methionines at residues 8 and 18, singlet *S*-methyl peaks appear at 2.08 and 2.10 ppm, whereas with the sulfoxide a single peak is seen at 2.70 ppm.

Unexpected signals in an NMR spectrum may arise from trivial low molecular weight contaminants, such as glycerol or acetic acid, rather than peptide byproducts. We have found that the use of the water eliminated Fourier transform (WEFT technique)[6] can rapidly distinguish trivial from peptidal impurities. For example, during the characterization of the purified PTH antagonist [Tyr^{34}]-bPTH(7–34)NH_2, the extraneous signal at 3.7 ppm was suppressed by this method (Figure 6), indicating it was a low molecular weight contaminant rather than a contaminating peptidal byproduct. This contaminant was subsequently identified by its spectrum to be glycerol, an observation confirmed by the use of an authentic sample.

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Conclusion

Fourier transform NMR spectroscopy, FAB mass spectroscopy, and peptide mapping have proven to be practical and powerful tools in the characterization of synthetic peptides and synthetic byproducts in the 4000-Da range. These techniques proved useful in permitting structure assignment to byproducts that could not have been readily characterized by more conventional methods, allowing more facile yield improvement for our parathyroid hormone analog program. Peptide mapping can also be employed to facilitate the chemical characterization of related compounds in an analogue program involving the synthesis of peptides of medium and large molecular weight.

Experimental Section

Peptide Synthesis. Automated solid-phase peptide synthesis was carried out on an Applied Biosystems, Inc., Model 430A peptide synthesizer with use of standard synthesis protocols provided in the ABI software for the instrument; 0.5 mmol of 4-methylbenzhydrylamine-polystyrene-co-(1% divinylbenzene) resin, HCl salt (pCH_3BHAR, 0.57 mmol N/g initial substitution, 0.877g) was neutralized with 10% diisopropylethylamine (DIEA) in *N,N*-dimethylformamide (DMF). After thorough washing with DMF, the first amino acid derivative protected by a *tert*-butyloxycarbonyl group (Boc) was coupled to the resin by using a preformed symmetric anhydride. α-Amino groups were protected with Boc, and the side chain protecting groups were tosyl for arginine, cyclohexyl for aspartic, benzyloxymethyl for histidine,[7] 2-chlorobenzyloxycarbonyl for lysine, bromobenzyloxycarbonyl for tyrosine, formyl for tryptophan, and benzyl for serine and glutamic acid. Symmetric anhydrides were formed in a separate reaction vessel by using 2 mmol of amino acid derivative and 1 mmol of dicyclohexylcarbodiimide (DCC) in CH_2Cl_2. After 10 min, precipitated dicyclohexylurea was filtered off, and CH_2Cl_2 was purged with a stream of N_2 and replaced with DMF. The preformed anhydride was added to the neutralized resin and allowed to couple for 20–45 min, depending on the residue. At the end of this time, the resin was washed extensively with CH_2Cl_2. Following repetition of the neutralization and coupling steps, a sample of the resin was removed for quantitative ninhydrin monitoring for completeness of the reaction and also for amino acid analysis.

The N-terminus of the resin-bound peptide was then deprotected with 60% trifluoroacetic acid in CH_2Cl_2, washed with CH_2Cl_2, and neutralized in preparation for the next coupling.

Arginine, glutamine, and asparagine were coupled as preformed 1-hydroxybenzotriazole esters as the acylating species by using 2 mmol of amino acid, 2 mmol of DCC, and 2 mmol of 1-hydroxybenzotriazole in DMF.

Cleavage. The peptide was cleaved from the resin with anhydrous HF. Analogues in which the Trp remained formylated were cleaved by using a 9:1 ratio of anhydrous HF/anisole (20 mL total volume/g dry resin). Completely deprotected Trp peptides were obtained by using a modification of the procedure of Tam et al.,[4] whereby the ratio of the carbonium ion scavengers *p*-cresol and *p*-thiocresol were changed to 5% each. (A mixture of formylated and deformylated Trp peptides was generated in our hands by using the unmodified procedure of 7.5% *p*-cresol and 2.5% *p*-thiocresol.)

Purification. Crude peptides from the HF cleavage procedure (typically 0.25–0.5 mmol) were initially desalted on a Sephadex G50F column (2 in. × 100 cm) directly after workup of the HF reaction. The column was eluted with 50% acetic acid; a late-eluting truncation byproduct peak was saved for further analysis, while the main peptide peak was combined, lyophilized, and then further purified by elution from a G50 Fractogel HW40F column

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(2 in. × 100 cm) with 2 N acetic acid. The main peptide containing portion of this column (in two batches of ~100/mg each) was then purified on a Waters Delta Prep. 3000 Preparative LC using a PrepPak reverse-phase C_{18} column (57 mm × 30 cm, 15–20 μm particle size, 300-Å pore size). A gradient of 15–30% B over 90 min at a flow rate of 100 mL/min (buffer A = 95% H_2O, 5% CH_3CN, 0.1% TFA; buffer B = 100% CH_3CN, 0.1% TFA) was used. Individual fractions were analyzed for purity by HPLC on a Spectra Physics 8000B LC using a C_4 4.2 mm × 10 cm, 5 μm particle size, 300-Å pore size column, 20% B to 35% A over 30 min.

Fractions containing ≥99.5% pure product by integration were combined, lyophilized, and then sent for further analysis.

Analytical Methods. Amino Acid Analysis. Amino acid analysis was performed under standard conditions on a Beckman System 6300. The amino acid analysis of Synthetic I is as follows [(theoretical):actual]. Lys(3):3.01; His(3):2.97; Arg(2):1.95, Asx(3):3.05; Ser(3):2.92; Glx(5):5.03; Gly(1):0.99; Val(2):1.99; Ile(1):0.95; Leu(4):4.12; Tyr(1):1.00; Phe(1):0.99; Nle(2):2.08.

Peptide Mapping. Two nanomoles of the peptide were digested with trypsin (100:1 by weight, in 0.02 M $NaHCO_3$, pH 8.5) at 37 °C for 4 h. The digest was immediately injected onto a Vydac C18 reverse-phase column, 150 × 4.6 mm equilibrated with 0.1% trifluoroacetic acid in water at 40 °C. The tryptic peptides were eluted with a linear gradient of 0–50% CH_3CN containing 0.1% TFA over 30 min at a flow rate of 1.5 mL per minute. The peaks of absorption at 210 nM were collected, and amino acid analyses were performed after 20-h acid hydrolyses.

NMR Spectroscopy. NMR spectra were obtained on a Varian XL-400 MHz spectrometer at ambient temperature with approximately 0.5 mM peptide. Chemical shifts are relative to internal deuteriated TSP (sodium 3-(trimethylsilyl)propionate-*2,2,3,3-d*$_4$). Suppression of the water signal was carried out by the WEFT sequence.[6]

FAB Mass Spectroscopy. The samples were analyzed by FAB-MS on a Finnigan-MAT No. 731 mass spectrometer fitted with an Ion Tek FAB gun by using xenon bombardment gas. Accelerating voltage was reduced from the normal 8 kV to 3 kV and resolution was set at R = 1000. Spectra were acquired in an SS 200 data system, and centroids of (M + H) peak clusters were interpolated between cesium iodide cluster ions to determine their masses.

Stereoselectivity in Electrophile-Mediated Intramolecular Cyclizations of Hept-2-enitols

Fillmore Freeman* and Kirk D. Robarge

Department of Chemistry, University of California, Irvine, Irvine, California 92717

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Electrophile (dibromine, diiodine, benzeneselenenyl chloride, and mercuric acetate) mediated intramolecular cyclization of (*Z*)-7-*O*-benzyl-1,2,3-trideoxy-4,5-*O*-isopropylidene-D-*ribo*-hept-2-enitol (**9**) gives predominantly 2,5-anhydro-1-*O*-benzyl-6,7-dideoxy-6-substituted-3,4-*O*-isopropylidene-D-*glycero*-L-*allo*-heptitol while electrophile-mediated intramolecular cyclization of (*E*)-7-*O*-benzyl-1,2,3-trideoxy-4,5-*O*-isopropylidene-D-*ribo*-hept-2-enitol (**11**) gives predominantly 3,6-anhydro-7-*O*-benzyl-1,2-dideoxy-2-substituted-4,5-*O*-isopropylidene-D-*glycero*-D-*gluco*-heptitol. The synthesis of D-*ribo*-hept-2-enitols **9** and **11** and the stereochemical and mechanistic aspects of their electrophile-mediated intramolecular cyclizations are discussed. The proposed Hehre reactivity model for electrophilic addition reactions to prochiral alkenes bearing an allylic oxygen predicts the stereochemical outcome of these kinetically controlled cyclizations.

Introduction

Since the pioneering work of Sinäy and coworkers,[1] electrophile-mediated cyclizations have been used in the formation of α- and β-2-deoxyhexapyranosides,[2] 6-deoxy-D-*xylo*-hex-5-enosides,[3] *C*-ribofuranosides,[4-7] *C*-arabinofuranosides,[8-10] *C*-nucleosides,[11-13] and natural products such as *N*-acetylneuraminic acid.[14] In the cases where five-membered rings (furan derivatives **2** and **4**) are formed from electrophilic attack onto an unsubstituted terminal olefin (**1** and **3**) and subsequent trapping by the internal nucleophile, there is usually an enrichment of the epimer in which H-2 and H-3 on the newly formed heterocycle are cis.[4-6,8-10] This results in the stereoselective formation of the α epimer of heterocycle **2** and the β epimer of heterocycle **4** (eq 1, 2). However, Mann and Kane[11] achieved excellent stereocontrol with ethyl (*Z*)-7-*O*-benzyl-2,3-dideoxy-4,5-*O*-isopropylidene-2-methyl-D-*ribo*-hept-2-enoate (**5**) to give solely the β epimer during the synthesis of showdomycin (eq 3). Freeman and Robarge[7] reported similar results with ethyl (*Z*)-7-*O*-benzyl-2,3-dideoxy-4,5-*O*-isopropylidene-D-*ribo*-hept-2-enoate (**7a**) and suggested that the origin of this β-selectivity in **5** may be due to a severe steric interaction in one conformer that is absent in the other (eq 4).[12]

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