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Brief Article

Synthesis, Conformational Analysis, and Biological Evaluation of 19-*nor*-Vitamin D_3 Analogues with A-Ring Modifications

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We have synthesized several isomers of 19-*nor*-vitamin D analogues possessing a hydroxy group at C-2 as well as novel derivatives bearing an epoxy substituent at the A-ring. All vitamins were prepared in convergent syntheses utilizing the modified Julia olefination. 1α,2α,25-Trihydroxy-19-*nor*-vitamin D_3 (**3**) and 2β,3β-epoxy-1α,25-dihydroxy-3-deoxy-19-*nor*-vitamin D_3 (**10**), which showed the highest affinity to the vitamin D receptor, displayed the highest potency among the tested compounds to inhibit the proliferation of MCF-7 breast cancer cells.

Introduction

First discovered as a treatment for the rickets disease in late 18th century, the efficacy of 1α,25-$(OH)_2$-D_3 (1α,25-$(OH)_2$-D_3 (**1**), Figure 1) as therapeutic agent in a variety of human diseases such as cancer, psoriasis, immunodeficiency, and osteoporosis is extremely promising.[1]

However, its clinical utility is limited because the therapeutically effective doses induce hypercalcemia and hypercalciuria. A continuing challenge in vitamin D research is to achieve an optimal balance between high antiproliferative and differentiation activities and low calcemic potency. During the last years, much attention has been focused on the synthesis of 19-*nor*-vitamin D derivatives. The effects of 1α,25-dihydroxy-19-*nor*-vitamin D_3 (**2**)[2] on HL-60 leukemia cell differentiation and osteocalcin secretion by MG63 cells were nearly identical to the natural hormone.[3b] Interestingly, its calcemic effects were clearly reduced (≤10%) compared to 1α,25-$(OH)_2$-D_3.[3] Recently, DeLuca and co-workers synthesized several 19-*nor* C-2 substituted derivatives, some of which have interesting biological activities.[4]

Synthesis of 1α,2α,25-trihydroxy-19-*nor*-vitamin D_3 (**3**, Figure 2) and 1α,2β,25-trihydroxy-19-*nor*-vitamin D_3 (**4**) have been reported.[5] The 2β-hydroxy analogue **4** had potent intestinal calcium transport equal to that of the native hormone, while the 2α-isomer **3** possessed less activity. The latter showed high potency in inducing differentiation of HL-60 cells with weak ability to mobilize calcium from bone.[5a]

Because of the importance of A-ring stereochemistry in the biological response, this paper aims to describe the synthesis of various 2-hydroxy derivatives (**5**−**8**) of the natural hormone with different stereochemistry at C-1, C-2, and C-3. In addition, the biological properties of these compounds were evaluated in order to compare with the corresponding 6-*s-cis* locked analogues previously described by us.[6] In this context, we also synthesized novel analogues containing an epoxy function at the A-ring (**9** and **10**).

Results and Discussion

Chemistry. The synthesis of compounds **5**−**10** was envisaged using the modified Julia olefination to construct a diene unit between the A-ring and the CD side chain fragment as previously reported by Kittaka.[5c]

A-Ring synthons were obtained starting from methyl quinate and its 3-*epi* and 5-*epi*-isomers[7] (Scheme 1). The next step was selective protection of the hydroxyl groups at C-3, C-4, and C-5 as *tert*-butyldimethylsilyl ethers. Reaction of the esters **12a**−**c** with $NaBH_4$ generated the alcohols **13a**−**c**, which were directly oxidized to cyclohexanone derivatives **14a**−**c** using $NaIO_4$.

The synthesis of the CD-ring/side chain fragment was accomplished starting from Grundmann's ketone, which was oxidized at the 25-position (steroid numbering) and then protected as an ethoxymethyl ether to afford **16** (Scheme 2).

The ketone was transformed to vinyl ester **17**, which was treated with DIBALH in toluene to provide the allylic alcohol **18**. The latter was converted to sulfone **19** through sulfonation with 2-mercaptobenzothiazole under Mitsunobu conditions and subsequent oxidation catalyzed by $(NH_4)_6MoO_{24}$.

A-Ring modified 19-*nor*-vitamin D_3 analogues were synthesized as illustrated in Scheme 3. Reaction of ketone **14a** with the lithium enolate of sulfone **19** and deprotection of the protecting groups with (−)-CSA gave 1α,2α,25-$(OH)_3$-19-*nor*-D_3 (**3**) and 1α,2β,25-$(OH)_3$-19-*nor*-D_3 (**4**) as a mixture of diastereoisomers in a 4:1 ratio.

Both analogues were separated by reverse phase HPLC. Since these derivatives have been previously described,[5] comparison of ^{1}H NMR data revealed that the major isomer was the 2α-hydroxy compound **3**, which was isolated in 52% yield (coupling and deprotection steps). Conformational equilibrium of **3** is strongly shifted to the α-chair conformer. On the other hand, compound **4** prefers a β-chair

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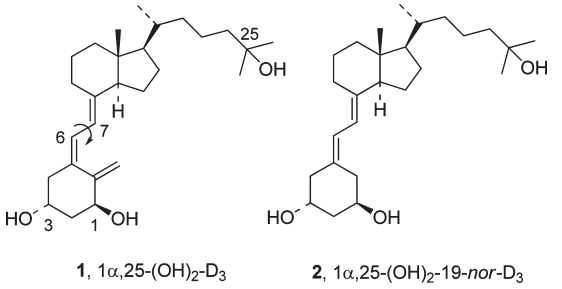


1, 1α,25-$(OH)_2$-D_3 **2**, 1α,25-$(OH)_2$-19-*nor*-D_3

Figure 1. Chemical structures of 1α,25-$(OH)_2$-D_3 and 1α,25-$(OH)_2$-19-*nor*-D_3.

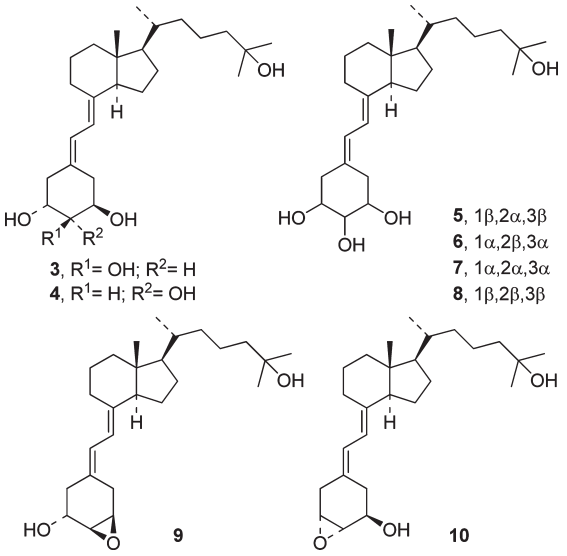
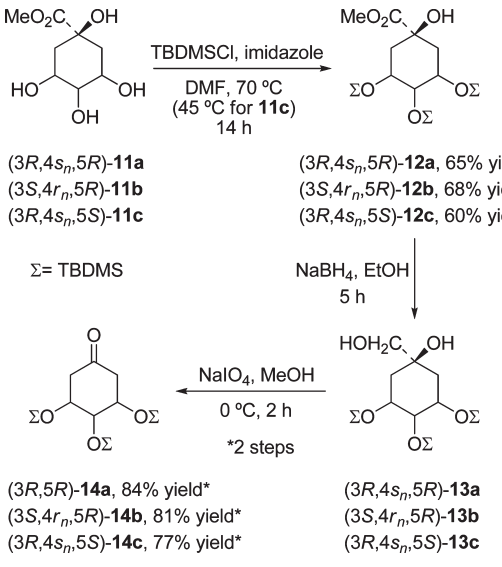


Figure 2. 2α- and 2β-1α,25-trihydroxy-19-*nor*-vitamin D_3 and new derivatives.

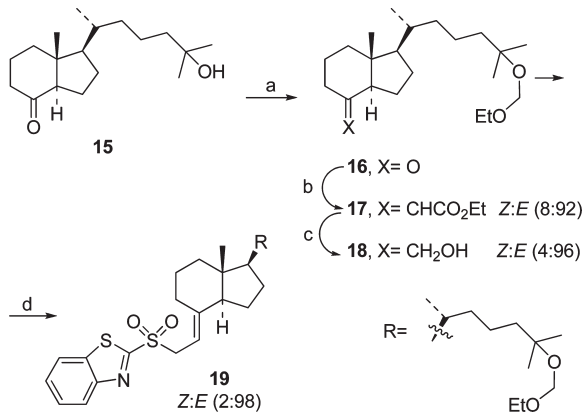
Scheme 1. Syntheses of A-Ring Precursors **14a**−**c**



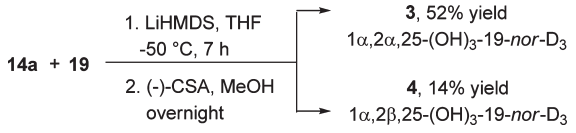
conformation (p S2, Supporting Information (SI)). This procedure constitutes an improvement on other reported methods.[5]

The coupling of ketone **14b** with sulfone **19** in similar conditions as above-described gave a 1:1 mixture of diastereoisomers **5** and **6** with moderate yield. At this stage, we called them **A** and **B** according to the rate of elution in the HPLC chromatogram (p S89, panel A, SI). The identification of the synthesized analogues was determined as follows.

Scheme 2. Synthesis of CD-Ring/Side Chain Fragment **19**



Scheme 3. Syntheses of 19-*nor*-Derivatives **3** and **4**



The protons at C1, C2, and C3 of derivative **A** appear close by in the ^1H NMR spectrum, and it was not possible to identify these signals. From their coupling constants, it is possible to determine the preferred chair conformation (Figure 3). In addition to the geminal constant, a coupling of 12 Hz in the axial H_4 proton was attributed to an axial−axial coupling to H_3, establishing the axial orientation of the latter. With these data, any of the chairs shown in Figure 3 could be from compound **A**.

In the case of compound **B**, signals for H_1 and H_3 overlap. The magnitude of the vicinal constants of H_2 ($^3J_{HH}$ 8.4, 8.4 Hz) indicates the axial disposition of H_1, H_2, and H_3. This is supported also by the coupling constants of H_1 and H_3 with the corresponding methylene adjacent protons. Thus, a coupling of 12 Hz between axial H_4 and H_3 establish their *trans* relationship.

These data indicate that both analogues exist in a chair conformation with the hydroxyl groups in equatorial disposition (chairs shown in Figure 3), but at this point we can not assign which chair corresponds to compound **A** or **B**. To identify the correct isomer, selected ROE experiments on H_6 and H_7 of both diastereoisomers were performed. In addition, optimized structures of compounds **A** and **B** calculated by hybrid DFT with GAUSSIAN-03 based on NMR data were obtained. From the data (p S2−S4, SI), it is possible to intuit that compound **A** corresponds to analogue **6** and therefore compound **B** is analogue **5**.

However, to deduce unequivocally the correct stereochemistry, an alternative synthesis of **5** and **6** were carried out. For that, quinate derivative **20**[7a] was transformed to ketone **22** (Scheme 4).

Coupling of ketone **22** with the anion generated from **19** followed by deprotection with aqueous trifluoroacetic acid give **5** and **6** as a mixture of diastereoisomers (approximately 3:7) in 65% yield (Scheme 5). In the HPLC chromatogram (p S89, panel B, SI), the major compound has higher rate of elution, previously assigned as **B**. Deprotection with

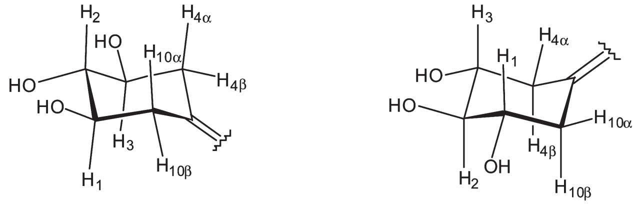
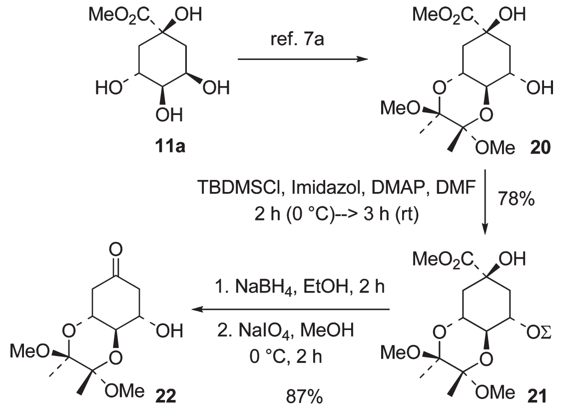
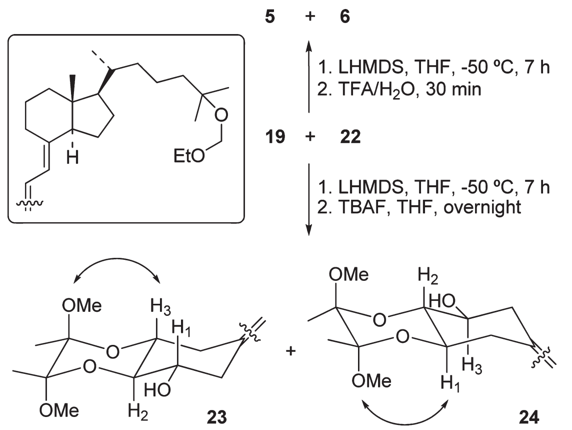


Figure 3. Preferred A-ring conformations for **5** and **6**.

Scheme 4. Synthesis of A-Ring Precursor **22**



Scheme 5. Syntheses and Selected ROEs of 19-*nor*-Derivatives **23** and **24**



TBAF instead of trifluoroacetic acid afforded compounds **23** and **24**.

ROESY spectrum of the mixture **23** and **24** shows in the major isomer a ROE between H_3 and one of the methoxy groups, which are oriented in the axial orientation to obtain the maximum anomeric stabilization. Therefore, the structure of this isomer is that depicted as compound **23** in Scheme 5. This proved that the major compound is **5**, as previously predicted by NMR experiments and calculations. Thus, A-ring chair conformation of **5** and **6** was unambiguously determined.

The vitamin D analogues **7** and **8** were obtained as a ca. 7:3 diastereoisomer mixture by coupling of **14c** with **19** as described above. Separation of these isomeric compounds was not possible.

There are scarcely examples of vitamin D analogues with an oxirane in its structure. Bouillon and co-workers reported some interesting vitamin D derivatives with epoxy functions in the side chain whose cell differentiating activity exceeded their calcemic effects more than 100-fold.[8] Because these derivatives of vitamin D show remarkable biological activities, epoxy A-ring analogues **9** and **10** were envisioned from the 1,2,3-triol moiety (C1, C2, C3) of preceding compounds **3−8** (Figure 2).

The A-ring epoxy precursor was prepared as depicted in Scheme 6. Starting from methyl quinate (**11a**), mesylate **25** was obtained in 64% yield.[9] Reduction of the ester to alcohol **26** and subsequent oxidation afforded ketone **27** in 50% overall yield.

Coupling of **19** and **27** with LHMDS, deprotection of all the protecting groups with (−)-CSA[a], and treatment with DBU in THF gave the isomeric epoxides **9** and **10** (ca. 3:2 diastereoisomeric mixture) in 53% yield. Both isomers were carefully separated by HPLC. Analysis of their coupling constants revealed the axial orientation of the 1α-hydroxyl group exhibited by the major isomer **10** (p S5, SI).

Biological Evaluation. The synthesized vitamin analogues were tested for their ability to bind the pig vitamin D receptor (VDR) and human vitamin D-binding protein (hDBP). A comparison between the natural hormone **1** and 2-hydroxy substituted analogues **3−8** indicates that derivatives with the natural configuration at C-1 and C-3 (**3** and **4**, Table 1) exhibited more potent affinity for the receptor than other isomers. The corresponding 2α-hydroxy analogue **3** displayed about 5 times less binding affinity than 1α,25-$(OH)_2$-D_3, while the 2*β*-hydroxy derivative **4** demonstrated 10-fold less binding affinity. The unnatural configuration at

Scheme 6. Synthesis of A-Ring Precursor **27**

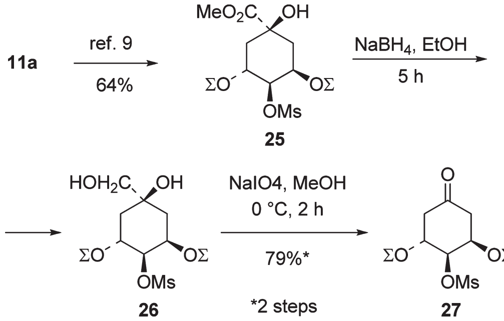


Table 1. In Vitro Effects of 19-*nor*-Vitamin D_3 Analogues on VDR and hDBP Binding and MCF-7 Cell Proliferation[a]

compd	VDR (%)	hDBP (%)	MCF-7 (%)
1α,25-$(OH)_2$-D_3	100	100	100
3	20	4	20
4	10	3	5
5	0.4	3	5
6	5	14	5
7 + 8	1	17	7
9	0.4	45	1
10	25	38	16

[a] The in vitro effect is expressed as percentage activity at EC_{50} in comparison with 1α,25-$(OH)_2$-D_3 (= 100% activity). The concentration of 1α,25-$(OH)_2$-D_3 necessary for half-maximal response in a [^{3}H-thymidine] incorporation assay on MCF-7 cells was $5.2 \pm 1.4 \times 10^{-8}$ M. 1α,25-$(OH)_2$-D_3 K_d for VDR: $0.7 \pm 0.02 \times 10^{-10}$ M. 1α,25-$(OH)_2$-D_3 K_d for DBP: $2.4 \pm 0.4 \times 10^{-8}$ M (in all cases, mean ± SD, $n = 3$).

[a] Abbreviations (−)-CSA, camphorsulfonic acid; VDR, vitamin D receptor; hDBP, human vitamin D binding protein; MCF-7, breast cancer cells; TBDMSCl, *tert*-butyldimethylsilyl chloride.

C-3 resulted in a decreased affinity for the VDR (**4** vs **6**, Table 1). In the case of the epoxy analogues, derivative **10** retained a significant binding affinity (25% with respect to the natural hormone), but the 1α,2α-epoxy analogue **9** was devoid of any affinity for VDR. These results indicated that vitamins with the axial orientation of the 1α-hydroxyl group exhibited the most potent affinity to VDR.

When hDBP binding of the 2-hydroxy analogues were compared, the 1α,2β,3α isomer **6** and the mixture **7** + **8** proved to possess more affinity. Interestingly, introduction of an epoxy function in the A-ring increased the binding potency. Both oxirane derivatives, **9** and **10**, showed 45% and 38% binding afffinity for hDBP, respectively, relative to the parent hormone. The lack of the C-1 hydroxyl group in **9** was not essential for binding. These epoxy analogues exhibited large differences in hDBP binding compared with the 2-hydroxy derivatives with the same stereochemistry in the A-ring (**9** vs **3** and **10** vs **4**, Table 1).

Next, the ability of these compounds to inhibit proliferation was examined in MCF-7 breast cancer cells. The 2α-hydroxy analogue **3** and the 2β,3β-epoxy derivative **10**, which showed the highest affinity to the VDR, displayed the highest potency among the tested compounds. Thus, at a concentration of 10^{-6} M, compound **3** can inhibit cell proliferation by 60%, which is comparable with 1α,25-$(OH)_2$-D_3 (p S7, Figure S7, SI). However, this derivative was 5 times less potent than 1α,25-$(OH)_2$-D_3 at the EC_{50} concentration. Similar results were observed with the epoxy analogue **10**. In contrast, epoxy **9** was almost unable to inhibit cell proliferation. In comparing antiproliferative activity of the 2α- and 2β-hydroxy derivatives (**3** and **4**), the 2α-analogue **3** exhibited a 4-fold increase in potency.

Comparison of the biological activity of these derivatives with our previous results of the corresponding 6-*s*-*cis* counterparts[6,10] possessing the same functionalities and stereochemistries showed that the 6-*s*-*trans* conformers displayed markedly increased binding affinity to VDR and hDBP and increased potency to inhibit MCF-7 cell proliferation.

Besides the in vitro screening, the in vivo calcemic effects of the compounds **3**, **9**, and **10** were evaluated. All tested analogues were less calcemic than 1α,25-$(OH)_2$-D_3 (p S7, Figure S8, SI). The tetraol analogue **3** demonstrated the weakest calcemic activity in mice because a daily injection of a dose exceeding 1000-fold the maximal tolerable dose of 1α,25-$(OH)_2$-D_3 (0.1 μg/kg/day) resulted in a smaller increase (13%) in serum calcium levels than 1α,25-$(OH)_2$-D_3 (27%).

Conclusions

We have described the synthesis and biological evaluation of 1α,25-dihydroxy-19-*nor*-vitamin D_3 analogues substituted at C-2 with a hydroxy group or possessing an epoxy functionality in the A-ring. To investigate structure−activity relationships, different stereochemistry at C-1, C-2, and C-3 were investigated. The structures of the analogues were determined by analysis of their coupling constants in addition to COSY and ROESY experiments. Results on VDR binding affinity and MCF-7 cell proliferation revealed that the axial orientation of the 1α hydroxyl group is necessary for biological activity. Comparison of the 2α- and 2β-hydroxy derivatives of 1α,25-dihydroxy-19-*nor*-vitamin D_3 showed that the 2α-isomer **3** has a higher VDR affinity than the corresponding 2β-isomer **4**. In vivo calcemic effects of synthesized analogues were evaluated showing very low calcemic effect.

Experimental Section

Chemistry. Synthesis of **11b**,[7a] **11c**,[7b] **20**,[7a] and **25**[9] were previously reported. The purity of compounds was determined in all cases as >99% by HPLC analysis.

General Procedure for 3−8. To a solution of **19** (43 mg, 0.078 mmol) in anhyd THF (260 μL) at −50 °C was added dropwise LHMDS (78 μL, 1.0 M in THF, 0.078 mmol) and the mixture was stirred at the same temperature for 2 h. Next, a solution of the corresponding ketone **14** (23 mg, 0.047 mmol) in anhyd THF (310 μL) was added dropwise to the mixture. After being stirred at −50 °C during 5 h, the reaction mixture was poured into a satd aq solution of NH_4Cl and extracted with Et_2O. The combined organic fractions were dried (Na_2SO_4), filtered, and concentrated to give a crude, which was dissolved in anhyd MeOH (1.2 mL) and cooled down at 0 °C. (−)-CSA (50 mg, 0.220 mmol) was then added, and the mixture was stirred a room temperature overnight. The reaction was quenched by adding a satd aq solution of $NaHCO_3$. The aqueous layer was extracted with EtOAc. The combined organic fractions were dried (Na_2SO_4), filtered, and concentrated to give a crude, which was purified by column chromatography using silica gel 60 Å (32−63 μm) pH 7 and EtOAc as eluent. The diastereoisomers were isolated by reverse-phase preparative HPLC (ODS-A column, 5 μm, 250 mm × 10 mm). Conditions: 4.5 mL/min, CH_3CN/H_2O (45:55) for **3** and **4**; 4 mL/min, CH_3CN/H_2O (50:50) for **5** and **6**. Retention times: t_R (**3**) = 28.6 min; t_R (**4**) = 24.1 min; t_R (**5**) = 20.7 min; t_R (**6**) = 18.9 min.

1α,2α-Epoxy-25-hydroxy-19-*nor*-vitamin D_3 (9) and 2β,3β-Epoxy-1α,25-dihydroxy-3-deoxy-19-*nor*-vitamin D_3 (10). Coupling of **19** (40 mg, 0.074 mmol) with **27** (20 mg, 0.044 mmol) and subsequent desilylation was performed through the same procedure as described above for the synthesis of **3**−**8**. To a solution of the resulting crude in anhyd THF (500 μL) was added dropwise DBU (10 μL, 0.066 mmol), and the reaction was stirred at room temperature for 16 h. Solvent was concentrated, and the crude was purified by column chromatography using silica gel 60 Å (32−63 μm) pH 7 and 10% hexane/EtOAc as eluent. The diastereoisomeric mixture was separated by reverse-phase preparative HPLC (ODS-A column, 5 μm, 250 mm × 10 mm, 5 mL/min, CH_3CN/H_2O 65:35). Retention times: t_R (**9**) = 20.4 min. t_R (**10**) = 23.4 min.

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Supporting Information Available: Experimental procedures, conformational analyses, energy-minimized conformations, in vitro and in vivo assays, NMR spectra; HPLC data. This material is available free of charge via the Internet at http://pubs.acs.org.

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