ORGANIC LETTERS

2003
Vol. 5, No. 14
2385−2388

Novel Sulfur-Containing Rapamycin Analogs Prepared by Precursor-Directed Biosynthesis§

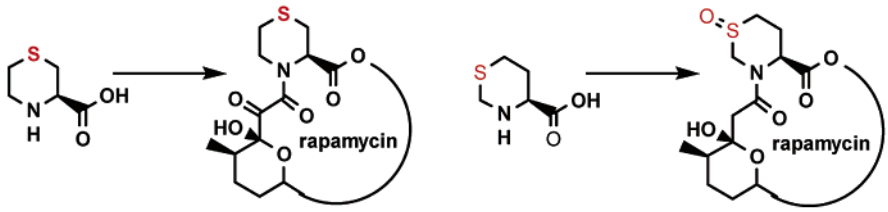
Edmund I. Graziani,*,† Frank V. Ritacco,† Mia Y. Summers,† T. Mark Zabriskie,‡ Ker Yu,† Valerie S. Bernan,† Michael Greenstein,† and Guy T. Carter†

Departments of Chemical & Screening Sciences and Oncology, Wyeth Research, 401 North Middletown Road, Pearl River, New York 10965, and Department of Pharmaceutical Sciences, Oregon State University, Corvallis, Oregon 97331

graziaei@wyeth.com

Received April 4, 2003

ABSTRACT



Two novel sulfur-containing analogs of the immunosuppressive natural product rapamycin (1) were obtained by feeding cultures of *Streptomyces hygroscopicus* with L-nipecotic acid (4) and either (*S*)-1,3-thiazane-4-carboxylic acid (5) or (*S*)-1,4-thiazane-3-carboxylic acid (6). The structures of the two new compounds, 20-thiarapamycin (2) and 15-deoxo-19-sulfoxylrapamycin (3), were determined by spectroscopic methods.

Rapamycin (**1**) is a hybrid polyketide/nonribosomal peptide macrolide first isolated from *Streptomyces hygroscopicus* NRRL 5491.[1] Over the past 25 years the potent antifungal activity[2] of **1** has been eclipsed by reports of its potent immunosuppressive and antiproliferative activities.[3,4] The compound is currently approved for use in renal transplantation (sirolimus, Rapamune, Wyeth Pharmaceuticals), and shows promise as a coating for coronary stents to prevent restenosis following angioplasty.[5] In addition, rapamycin derivative CCI-779 is undergoing clinical trials for a number of tumor types.[6] Moreover, this remarkable compound effects these activities via an exquisite protein−small molecule−protein interaction.[7]

While the compound has been the object of many total syntheses as well as the subject of much semisynthetic structure−activity work,[8] there remains considerable scope for preparing biologically active analogs of rapamycin that are inaccessible by routes other than total synthesis. Rapamycin analogs have been made previously by precursor-directed biosynthesis and P-450 inhibitor addition,[9] biotransformation,[9,10] and genetic manipulation.[11]

§ This paper is dedicated to the memory of Dr. Suren Sehgal, 1932−2003.

† Wyeth Research.

‡ Oregon State University.

(1) Sehgal, S. N.; Baker, H.; Vézina, C. *J. Antibiot.* **1975**, *28* (10), 721−726.
(2) Singh, K.; Sun, S.; Vézina, C, *J. Antibiot.* **1979**, *32* (6), 630−645.
(3) Sehgal, S. N. *Clin. Biochem.* **1998**, *31*(5), 335−340.
(4) Abraham, R. T. *Cell* **2002**, *111*, 9−12.
(5) Marx, S. O.; Marks, A. R. *Circulation* **2001**, *104* (8), 852−855.
(6) Hidalgo, M.; Rowinsky, E. K. *Oncogene* **2000**, *19*, 6680−6686.
(7) Choi, J.; Chen, J.; Schrieber, S. L.; Clardy, J. *Science* **1996**, *273*, 239−241.
(8) For reviews see: (a) Farooq, A.; Anjum, S.; Ur-Rahman, A. *Curr. Org. Chem.* **1998**, *2*, 281−328. (b) Caufield, C. E. *Curr. Pharm. Des.* **1995**, *1* (2), 145−160.
(9) Nishida, H.; Sakakibara, T.; Aoki, F.; Saito, T.; Ichikawa, K.; Inagaki, T.; Kojima, Y.; Yamauchi, Y.; Huang, L. H.; Guadliana, M. A.; Kaneko, T.; Kojima, N. *J. Antibiot.* **1995**, *48* (7), 657−666.
(10) Chen, T. S.; Arison, B. H.; Wocker, L. S.; Inamine, E. S.; Monaghan, R. L. *J. Antibiot.* **1992**, *45* (1), 118−123.

10.1021/ol034591k CCC: $25.00
Published on Web 06/17/2003

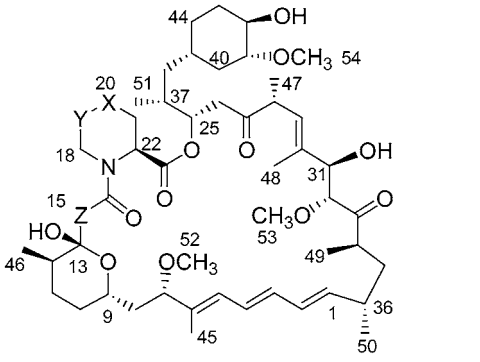


Figure 1. Structures of rapamycin (**1**), 20-thiarapamycin (**2**), and 15-deoxo-19-sulfoxylrapamycin (**3**).

We undertook to modify the pipecolate ring of rapamycin by inhibiting L-pipecolate production in the producing organism. It has been shown that proline analogs can inhibit production of rapamycin, presumably by inhibiting the lysine cyclodeaminase activity of the *rapL* gene product, since rapamycin production is restored by co-addition of L-pipecolic acid.[12] This observation suggested that incorporation of unusual pipecolic acid analogs was possible, provided they could serve as substrates for the pipecolate-incorporating enzyme (PIE) coded by *rapP*. This enzyme is responsible for condensing the adenylpipecolate with the fully elaborated polyketide chain produced by the upstream PKS, in addition to catalyzing the macrolactonization step. Previous work on the enzymology of pipecolate incorporation into the related immunosuppresant, FK520, indicated the ability of PIE to accommodate a number of substrate analogs.[13] The anticipated relaxed substrate specificity of the PIE is further indicated by production of 4-hydroxyproline analogs of rapamycin in a *rapL* mutant strain of *S. hygroscopicus*.[11]

Having screened a number of potential *rapL* gene product inhibitors, we determined that (±)-nipecotic acid **(4)** inhibited production of rapamycin by *S. hygroscopicus*, with prolylrapamycin[14] being the major metabolite present; concurrent feeding of L-pipecolate restored rapamycin production.[15] Two sulfur-containing pipecolate analogs, (*S*)-1,4-thiazane-3-carboxylic acid (**5**) and (*S*)-1,3-thiazane-4-carboxylic acid (**6**), were prepared by literature methods[16] and fed to cultures of *S. hygroscopicus* along with **4**, yielding two novel rapamycin analogs as a result.

(11) Khaw, L. E.; Böhm, G. A.; Metcalfe, S.; Staunton, J.; Leadlay, P. F. *J. Bacteriol.* **1998**, *180* (4), 809−814.
(12) Kojima, I.; Demain, A. L. *J. Ind. Microbiol. Biotechnol.* **1998**, *20*, 309−316.
(13) Nielsen, J. B.; Hsu, M. J.; Byrne, K. M.; Kaplan, L. *Biochemistry* **1991**, *30*, 5789−5796.
(14) Russo, R. J.; Howell, S. R.; Sehgal, S. N. U.S. Patent 5441977, 1995.
(15) A detailed account of the screening of inhibitors and fermentation experiments will be published elsewhere.

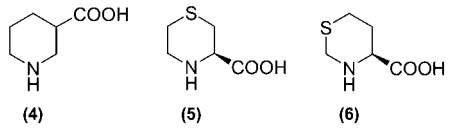


Figure 2. Structures of (±)-nipecotic acid (**4**), (*S*)-1,4-thiazane-3-carboxylic acid (**5**), and (*S*)-1,3-thiazane-4-carboxylic acid (**6**).

Cultures of *S. hygroscopicus* were fed **4** and **5** and 20-thiarapamycin (**2**, 2 mg from 20 mL of culture = 100 mg/L) was obtained via purification of whole-cell extracts by reversed-phase chromatography.[17] FT-ICRMS analysis yielded the molecular formula $C_{50}H_{77}NO_{13}S$ for **2**, which exactly matched that predicted for intact incorporation of the 1,4-thiazane. Although the NMR assignments of **2** were complicated by the two conformational populations observed due to cis/trans isomerization of the amide bond,[18] all resonances for the major conformer were assigned and are in good agreement with the literature.[19]

The important NMR correlations that confirmed the assignments for the thiazane ring of **2** are diagrammed in Figure 3. Although the chemical shifts of the lactone and amide carbonyls are almost overlapped, it was possible to distinguish an HMBC correlation from the α-proton H22 (δ 5.27 ppm) into the amide carbonyl at δ 167.4 ppm. This amide carbonyl resonance showed an additional HMBC correlation from one of the H18 methylene protons at δ 3.71 ppm. The carbon and proton NMR chemical shifts for C18 were obtained from the multiplicity edited me-HSQC spectrum[20] of **2**, and were consistent for a methylene adjacent

(16) **5**: Carson, J. F.; Wong, F. F. *J. Org. Chem.* **1964**, *29*, 2203−2205. **6**: Miyazaki, H.; Ohta, A.; Kawakatsu, N.; Waki, Y.; Gogun, Y.; Shiraiwa, T.; Kurokawa, H. *Bull. Chem. Soc. Jpn.*, **1993**, *66* (2), 536−540.
(17) 20-Thiarapamycin (**2**): amorphous white solid; UV (MeOH) 268, 278, 288 nm; FT-ICRMS *m/z* 954.50001 for $C_{50}H_{77}NO_{13}SNa$ $(M + Na)^+$ (calcd 954.501333; Δ mmu = 1.32); ^{1}H NMR (500 MHz, d_6-DMSO) 6.54 (s 13-OH), 6.39 (dd, 14.0, 11.9; H4), 6.20 (H3), 6.15 (H2), 6.09 (H5), 5.43 (dd, 14.3, 9.6; H1), 5.27 (H22), 5.26 (31-OH), 5.12 (d 9.9; H29), 4.97 (H25), 4.11 (d 4.4; 42-OH), 3.99 (H9), 3.98 (H31), 3.83 (dd 5.2, 1.3; H32), 3.71 (br d 15.2; H18), 3.61 (br d 11.9; H7), 3.48 (H18′), 3.30 (3H, s H54), 3.29 (H28), 3.16 (H42), 3.13 (3H, s H53), 3.03 (3H, s H52), 2.95 (H21), 2.92 (H21′), 2.82 (H41), 2.74 (d 16; H26), 2.60 (H19), 2.55 (H19′), 2.47 (H34), 2.45 (H26′), 2.22 (H36), 2.03 (H12), 1.92 (H40), 1.88 (H8), 1.81 (H10), 1.74 (H43), 1.70 (H37), 1.69 (3H, s H48), 1.63 (3H, s H45), 1.53 (H44), 1.51 (H11), 1.49 (H11′), 1.40 (H35), 1.29 (H8′), 1.27 (H39), 1.18 (H10′), 1.17 (H43′), 1.08 (H38), 1.05 (H35′), 0.97 (H38′), 0.96 (3H, d 5.0; H50), 0.90 (3H, d 6.1; H47), 0.86 (H44′), 0.84 (3H, d 5.6; H49), 0.76 (3H, d 6.1; H51), 0.72 (3H, d 6.3; H46), 0.58 (H40′); ^{13}C NMR (125 MHz, d_6-DMSO) 210.8 (s, C33), 207.6 (s, C27), 199.1 (s, C15), 167.4 (s, C16), 167.0 (s, C23), 139.1 (d, C1), 137.8 (s, C6), 137.6 (s, C30), 132.2 (d, C3), 129.9 (d, C2), 126.7 (d, C4), 126.6 (d, C5), 124.9 (d, C29), 99.1 (s, C13), 85.6 (d, C32), 83.6 (d, C41), 82.2 (d, C7), 75.5 (d, C31), 74.3 (d, C25), 73.0 (d, C42), 66.4 (d, C9), 56.9 (q, C53), 56.6 (q, C54), 55.2 (q, C52), 50.7 (d, C22), 45.1 (d, C28), 44.7 (t, C18), 40.0 (t, C26), 39.7 (t, C8), 39.1 (d, C34), 39.0 (t, C35), 38.3 (t, C38), 35.2 (d, C36), 35.2 (t, C40), 34.9 (d, C12), 33.5 (d, C37), 32.9 (t, C43), 32.6 (d, C39), 31.0 (t, C44), 29.4 (t, C10), 27.8 (t, C21), 26.3 (t, C11), 26.0 (t, C19), 15.6 (q, C50), 15.4 (q, C46), 14.4 (q, C51), 14.0 (q, C49), 12.8 (q, C48), 10.4 (q, C45).
(18) Kessler, H.; Haessner, R.; Schüler, W. *Helv. Chim. Acta* **1993**, *76*, 117−130.
(19) (a) McAlpine, J. B.; Swanson, S. J.; Jackson, M.; Whittern, D. N. *J. Antibiot.* **1991**, *44*(6), 688−690. (b) Findlay, J. A.; Radics, L. *Can. J. Chem.* **1980**, *58*, 579−590.
(20) Willker, W.; Leibfritz, D.; Kerssebaum, R.; Bermal, W. *Magn. Reson. Chem.* **1993**, *31* (3), 287−292.

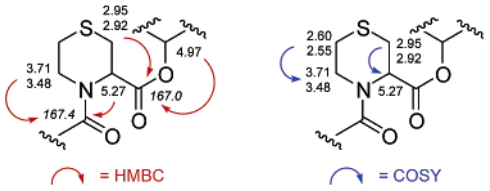


Figure 3. Selected HMBC and COSY correlations for 20-thiarapamycin (**2**).

to nitrogen. The COSY spectrum of **2** revealed that the H18 protons were part of a spin system that contained only two additional members, two closely overlapping resonances at δ 2.60 and 2.55 ppm that were both attached to a carbon resonance at δ 26.0 ppm assigned to C19. Similarly, H22 showed only one coupling partner in the COSY spectrum of **2**, a set of overlapping signals at δ 2.95 and 2.92 ppm that were correlated into a carbon resonance at δ 27.8 ppm in the me-HSQC spectrum of **2**, and were assigned to the H21 protons.

In another experiment, 15-deoxo-19-sulfoxylrapamycin **(3**, 10 mg from 1 L of culture = 10 mg/L) was obtained from cultures of *S. hygroscopicus* fed **4** and **6** via purification of fermentation extracts by reversed-phase chromatography.[21] In contrast to **2**, the molecular formula given by high-resolution mass measurements of **3**, $C_{50}H_{79}NO_{13}S$, did not fit with the expected formula for intact incorporation of 1,3-thiazane ($C_{50}H_{77}NO_{13}S$, as per **2**), providing a formal surfeit of two hydrogens. Examination of the NMR data for **3** revealed an unanticipated set of methylene protons at δ 2.99 and 2.55 ppm in the me-HSQC spectrum of **3** that showed correlations into a carbon at δ 43.1 ppm. These two methylene protons also showed HMBC correlations into a resonance at δ 97.9 ppm corresponding to the hemiketal carbon C13. These methylene proton resonances showed further HMBC correlations into a carbonyl signal at δ 170.6, indicating that these signals must be assigned to a methylene

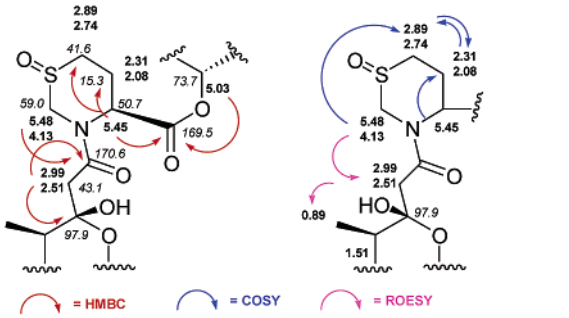


Figure 4. Selected HMBC, COSY, and ROESY correlations for 15-deoxo-19-sulfoxylrapamycin (**3**).

group at C15. These assignments are in good agreement with the values reported previously for rapamycin analogs in which C15 is reduced to a fully saturated methylene group.[9] This accounts for the previously mentioned surfeit of two hydrogens in the molecular formula of **3** relative to **2**, although formal accounting of atoms now requires an additional oxygen elsewhere in the structure of **3**.

The NMR resonance for the amide carbonyl C16 at δ 170.6 shows additional correlations in the HMBC spectrum of **3** from proton signals at δ 5.48 and 4.18 ppm. These resonances both correlated into a carbon signal at δ 59.0 ppm in the me-HSQC spectrum of **3**. This methylene group was therefore assigned to C18. It was noted that the proton and carbon chemical shifts were considerably more downfield for this position than in either rapamycin or **2**. This observation suggested the possibility that the sulfur presumed to be incorporated at position 19 had been oxidized to the corresponding sulfoxide. The chemical shifts for the methylene protons at C18 were in good agreement with those reported for proline *S*-oxide analogs.[22] Interestingly, the observed chemical shift for C21 (δ 15.3 ppm) is considerably more upfield than would be expected for a methylene carbon, and can best be accounted for by the shielding effect of the sulfinyl group.[23] This assignment furthermore accounts for the remaining oxygen required by the molecular formula, given that the rest of the molecule is intact relative to rapamycin (see assignments[21]). The other assignments for the *S*-oxide pipecolate ring were made using COSY, HMBC, and ROESY correlations, as shown in Figure 4.

The stereochemistry of the sulfoxide moiety can also be inferred from the NMR data. The large upfield shift for C21 in combination with the observed deshielding of the axial proton H21 (δ 2.31 ppm), relative to H5 axial in **6** (δ 1.52 ppm),[24] support the assignment of the more stable pseudo-axial β-sulfoxide.[25,26]

(21) 15-Deoxo-19-sulfoxylrapamycin (**3**): amorphous white solid; UV (MeOH) 268, 278, 288 nm; FT-ICRMS *m*/*z* 956.51631 for $C_{50}H_{79}NO_{13}$-SNa ($M + Na)^+$ (calcd 956.516983; Δ mmu = 0.673); ^{1}H NMR (500 MHz, d_6-DMSO) 6.41 (dd 14.5, 11.3; H4), 6.26 (H3), 6.21 (H5), 6.17 (H2), 5.48 (H18), 5.46 (H1), 5.45 (H22), 5.10 (d 10.2; H29), 5.03 (H25), 4.18 (H18′), 4.17 (H32), 4.06 (br s H31), 3.94 (H9), 3.63 (H7), 3.32 (3H, s H54), 3.20 (H28), 3.17 (3H, s H53), 3.16 (H42), 3.05 (3H, s H52), 2.99 (br d 14.6; H15), 2.89 (H20), 2.81 (H41), 2.74 (br d 11.4; H20′), 2.68 (H26), 2.51 (H15′), 2.36 (dd 16.5, 2.4; H26′), 2.31 (H21), 2.25 (H34), 2.25 (H36), 2.08 (H21′), 1.94 (H40), 1.88 (H8), 1.80 (3H, s H48), 1.75 (H10), 1.74 (H43), 1.68 (H37), 1.62 (3H, s H45), 1.53 (H44), 1.51 (H12), 1.49 (H11), 1.41 (H35), 1.39 (H11′), 1.21 (H39), 1.16 (H43′), 1.07 (H38), 1.04 (H10′), 1.02 (H8′), 1.00 (H35′), 0.99 (3H, d 6.5; H50), 0.95 (H38′), 0.89 (3H, H46), 0.81 (H44′), 0.81 (3H, d 6.4; H47), 0.76 (3H, d 6.1; H49), 0.76 (3H, d 6.1; H51); ^{13}C NMR (125 MHz, d_6-DMSO) 209.4 (s, C33), 208.8 (s, C27), 170.6 (s, C16), 169.5 (s, C23), 138.8 (d, C1), 137.7 (s, C6), 137.0 (s, C30), 132.2 (d, C3), 130.4 (d, C2), 127.3 (d, C5), 127.0 (d, C4), 123.3 (d, C29), 97.9 (s, C13), 84.5 (d, C32), 83.6 (d, C41), 82.2 (d, C7), 75.9 (d, C31), 73.7 (d, C25), 72.9 (d, C42), 65.8 (d, C9), 59.0 (t, C18), 56.5 (q, C54), 56.4 (q, C53), 55.2 (q, C52), 50.7 (d, C22), 45.5 (d, C28), 43.1 (t, C15), 41.6 (t, C20), 40.8 (d, C34), 40.2 (t, C8), 39.6 (t, C26), 39.0 (t, C35), 38.4 (t, C38), 35.7 (d, C12), 35.4 (t, C40), 34.7 (d, C36), 33.4 (d, C37), 32.8 (t, C43), 32.5 (d, C39), 30.7 (t, C44), 30.1 (t, C10), 26.9 (t, C11), 24.3 (q, C50), 16.5 (q, C46), 15.3 (t, C21), 15.2 (q, C47), 14.5 (q, C48), 12.0 (q, C49), 11.9 (q, C51), 10.1 (q, C45).

(22) Kánai, K.; Podányi, B.; Bokotey, S.; Hajdú; Hermecz, I. *Tetrahedron*: *Asymmetry* **2002**, *13*, 491−495.

(23) McCrachren, S. S.; Evans, S. A. *J. Org. Chem.* **1979**, *44* (20), 3551−3555.

(24) The ^{1}H NMR spectrum of **6** was measured in d_6-DMSO; cf.: Zabriskie, T. M.; Liang, X. *Bioorg. Med. Chem. Lett.* **1997**, *7* (4), 457−462.

(25) Frieze, D. M.; Hughes, P. F.; Merrill, R. L.; Evans, S. A. *J. Org. Chem.* **1977**, *42* (13), 2206−2211.

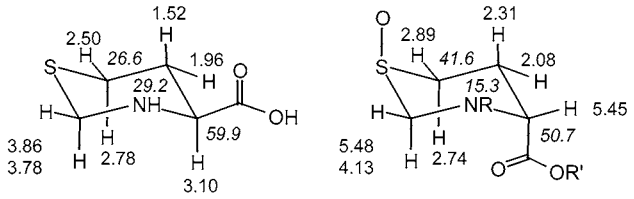


Figure 5. Comparison of idealized chair conformations of the thiazane ring of **6** (left) and **3** (right) illustrating the pseudoaxial sulfoxide configuration based upon the downfield shift of the axial H21 proton chemical shift.

The biological activities of rapamycin are dependent upon the binding of rapamycin to FKBP12 and further binding of this complex to the target-of-rapamycin protein, mTOR.[27] It has been shown previously that the pipecolate ring and its flanking region are critical for FKBP12 binding, with important hydrogen bonds involving carbonyls at C15, C16, and C23 of rapamycin.[28] 20-Thiarapamycin (**2**) had an IC_{50} of 54 nM in an FKBP12 binding assay, indicating that the compound is 2 orders of magnitude less tightly bound to this protein than rapamycin. Unsurprisingly, 15-deoxo-19-sulfoxyl-rapamycin (**3**) was an even weaker binder of FKBP12, with an IC_{50} of 800 nM.

With respect to the biosynthesis of **2** and **3**, it is interesting that 1,4-thiazane-3-carboxylic acid (**5**) is incorporated by the PIE into rapamycin with no further downstream perturbation of biosynthetic enzymes to yield the fully elaborated 20-thiarapamycin (**2**). In contrast, incorporation of 1,3-thiazane-4-carboxylic acid (**6**) occurs with a much lower overall yield (10 mg/L vs 100 mg/L for **2**) and with considerable perturbation of the cytochrome P-450 monooxygenase that elaborates the carbonyl at C15 in rapamycin.[29] The detection of **3** in fresh extracts of *S. hygroscopicus* that had been fed **6** suggests that sulfoxide formation is not an isolation artifact. It is more likely that **6** serves as a substrate for the PIE and that the sulfur is oxidized after incorporation, presumably prior to late-stage P-450 hydroxylation events, though additional work will be required to further confirm this.

In conclusion, two novel sulfur-containing analogs of the natural product rapamycin have been prepared by precursor directed biosynthesis. Extensive spectroscopic analyses revealed that one analog, **2**, showed intact incorporation of the precursor, **5**, into the structure of rapamycin. In contrast, incorporation of **6** led to the isolation of the unexpected variant, **3**, in which the sulfur was present as a sulfoxide and in which a late-stage cytochrome P-450 oxidation had been inhibited. In both cases, the binding of **2** and **3** to FKBP12 was weaker than that of rapamycin itself, with the larger perturbations in the FKBP12 binding region of **3** causing a marked decrease in binding.

Acknowledgment. We thank Dr. Xidong Feng for FT-ICRMS analysis and Dr. R. Thomas Williamson for acquiring NMR data and for helpful suggestions in preparing this paper.

Supporting Information Available: ^{1}H, COSY, me-HSCQC, and HMBC NMR spectra for **2** and ^{1}H, COSY, me-HSCQC, HSQC-TOCSY, ROESY, and HMBC NMR spectra for **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

OL034591K

(26) Hutton, C. A.; Jaber, R.; Otaegui, M.; Turner, J. J.; Turner, P.; White, J. M.; Bacskay, G. B. *J. Chem. Soc., Perkin Trans. 2* **2002**, 1066−1071.
(27) Abraham, R. T. *Curr. Opin. Immunol.* **1998**, *10* (3), 330−336.
(28) Van Duyne, G. D.; Standaert, R. F.; Schrieber, S. L.; Clardy, J. *J. Am Chem. Soc.* **1991**, *113*, 7433−7434.
(29) Molnár, I.; Aparicio, J. F.; Haydock, S. F.; Khaw, L.; Schwecke, T.; König, A.; Staunton, J.; Leadlay, P. F. *Gene* **1996**, *169*, 1−7.