DOI: 10.1002/cbic.201300096

In Vivo Probing of the Temperature Responses of Intracellular Biomolecules in Yeast Cells by Label-Free Raman Microspectroscopy

Yu-Fang Chiu, Chuan-Keng Huang, and Shinsuke Shigeto*[a]

Environmental temperature is an essential physical quantity that substantially influences cell physiology by changing the equilibria and kinetics of biochemical reactions occurring in cells. Although it has been extensively used as a readily controllable parameter in genetic and biochemical research, much remains to be explored about the temperature responses of intracellular biomolecules in vivo and at the molecular level. Here we report in vivo probing, achieved with label-free Raman microspectroscopy, of the temperature responses of major intracellular components such as lipids and proteins in living fission yeast cells. The characteristic Raman band at 1602 cm^{-1}, which has been attributed mainly to ergosterol, showed a significant decrease ($\approx$47%) in intensity at elevated temperatures above 35 °C. In contrast to this high temperature sensitivity of the ergosterol Raman band, the phospholipid and protein Raman bands did not vary much with increasing culture temperature in the 26–38 °C range. This finding agrees with a previous biochemical study that showed that the initial stages of ergosterol biosynthesis in yeast are hindered by temperature elevation. Moreover, our result demonstrates that Raman microspectroscopy holds promise for elucidation of temperature-dependent cellular activities in living cells, with a high molecular specificity that the commonly used fluorescence microscopy cannot offer.

Introduction

Temperature profoundly affects a plethora of physiological processes that take place in living cells, such as cell division, metabolism, protein transport, and cell death. This high sensitivity of physiological processes to temperature arises from the fact that the equilibria and kinetics of individual biochemical reactions involved in the physiological processes are strongly temperature-dependent. Along with pH and oxygen concentration, temperature has been widely used as a critical parameter in genetic and biochemical experiments. Temperature-sensitive mutation, for example, has substantially advanced understanding of the molecular mechanisms of many important biological processes.[1–4] We still know little, however, about how cells respond to temperature variation in vivo and at the molecular level and what distinct behavior patterns intracellular components show at various temperatures.

In this study we discuss the temperature responses of major intracellular biomolecules in living fission yeast (*Schizosaccharomyces pombe*) cells probed by Raman microspectroscopy. Unlike most biochemical approaches, microscopic techniques can investigate single living cells in a nondestructive manner. Fluorescence microscopy with the use of fluorescent nanothermometers based on lanthanide nanoparticles,[5,6] quantum dots,[7] or polymer-embedded organic dyes[8–11] has recently permitted accurate measurements of intracellular temperature in living cells. Of particular importance is the work done by Okabe et al.,[9] which for the first time has extended intracellular temperature measurements at specific locations to mapping, with a temperature resolution of <1 K.

In contrast, the focus of this work is on the effects of environmental temperature (i.e., culture temperature in this case) rather than on intracellular temperature. Whereas intracellular temperature is closely associated with cellular thermogenesis[12,13] (i.e., local heat generation as a consequence of cellular activities), environmental temperature alters the rate of the metabolic activity of cells and thereby causes changes in cell proliferation rate. By analyzing the fingerprint Raman spectra (600–1800 cm^{-1}) of fission yeast cells measured at culture temperatures ranging from 26.1 to 38.3 °C, we show that the biosynthesis of the fungal sterol ergosterol[14]—in contrast with the less temperature-sensitive behavior of proteins and phospholipids—is significantly diminished at culture temperatures higher than 35 °C. This study demonstrates that Raman microspectroscopy can be added to the toolbox for in vivo molecular-specific probing of temperature-dependent cellular activities without the need to introduce fluorophores, which might perturb the physiological state of cells.

Results and Discussion

We used a laboratory-built confocal Raman microspectrometer[15–17] equipped with a microscope stage-top incubator to record Raman spectra of 30 fission yeast cells under the microscope at ten different temperatures ranging from 26.1 to 38.3 °C. The cells were precultured in Yeast and Mold (YM) broth in a shaker incubator and then transferred to a glass-bot-

[a] Y.-F. Chiu, C.-K. Huang, Prof. Dr. S. Shigeto
Department of Applied Chemistry and Institute of Molecular Science
National Chiao Tung University
1001 Ta-Hsueh Road, Hsinchu 30010 (Taiwan)
E-mail: shigeto@mail.nctu.edu.tw

tomed dish for Raman measurements (see the Experimental Section for details). The growth curves of fission yeast cells at optimum temperature[18] (30 °C) and at 38 °C are compared in Figure 1 A. After 40 h, the cells had reached stationary phase

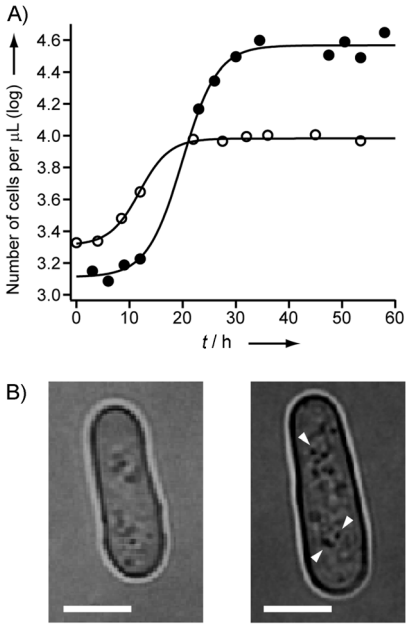

Figure 1. A) Growth curves of fission yeast cells cultured in YM broth at 30 (●) and 38 °C (○). Solid lines are sigmoid fits provided as a guide for the eye. B) Bright-field optical images of typical living yeast cells cultured at 30 (left) and 38 °C (right). White arrowheads indicate the intracellular locations of several lipid droplets (dark spots). Scale bars: 5 μm.

even at 38 °C, but the number of yeast cells cultured at this adverse temperature was not as large as that at 30 °C. No noticeable difference between the bright-field optical images of typical living yeast cells grown at 30 and 38 °C was found (Figure 1 B). With 632.8 nm excitation at 2.6 mW excitation power (see the Experimental Section), the target yeast cell tended to be optically trapped. Optical trapping (also known as optical tweezers) is a powerful technique for capturing and manipulating biological particles at the laser focus.[19] It facilitates both efficient Raman excitation and collection of Raman scattered light in a confocal configuration.[20–22]

Figure 2 displays a series of averaged Raman spectra of optically trapped yeast cells at 10 different culture temperatures. Each spectrum is the average of 30 background-subtracted spectra and has been normalized to the peak area of the CH bending band at 1440 cm^{-1}. The use of the peak area of the 1440 cm^{-1} band as a normalization standard is justified because it has been found to remain almost constant over the whole temperature range studied.

A number of characteristic Raman bands can be seen in Figure 2. Vibrational assignments of those bands, except for the band at 1602 cm^{-1}, have been extensively discussed in the literature,[23–25] as summarized below. The weak band at 1744 cm^{-1} is assigned to the C=O stretch of the ester moieties of phospholipids. The band at 1655 cm^{-1} is attributed to the C=C stretch of the *cis* –CH=CH– linkages of unsaturated lipid

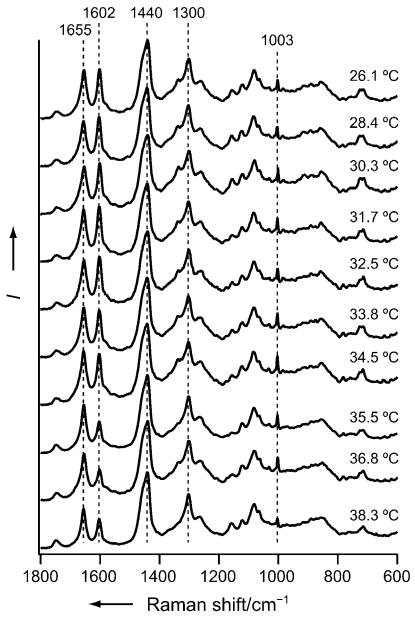

Figure 2. Averaged Raman spectra (fingerprint region), of fission yeast cells cultured at 10 different temperatures. Each spectrum is the average of 30 Raman spectra obtained from 30 different randomly chosen yeast cells. All the spectra have been normalized to the peak area of the CH bending band at 1440 cm^{-1}.

chains. As described above, the prominent 1440 cm^{-1} band is assigned to the CH bending modes, including CH_2 scissors and CH_3 degenerate deformation. The band at 1300 cm^{-1} is assigned to the in-plane CH_2 twisting mode. All these Raman bands are thought to be predominantly due to lipids, because the yeast cells are likely to be trapped at lipid droplets (dark spots indicated by arrowheads in Figure 1 B), extremely lipid-rich organelles. Possible contributions from overlapping protein bands (e.g., amide I) are therefore considered minor. The sharp peak at 1003 cm^{-1} is a protein Raman band arising from the ring-breathing mode of the phenylalanine residues.

The band at 1602 cm^{-1} is an interesting Raman signature of yeast and has been intensively studied by Hamaguchi and coworkers. They examined the behavior of the 1602 cm^{-1} band on treatment of the cells with respiration inhibitors such as KCN[24] and NaN_3,[26] under different nutrition and atmospheric conditions[27] and in the presence of oxidative stress induced by addition of H_2O_2.[27] Despite these thorough investigations, the origin of the band had long been unclear. Very recently, however, it has been shown that ergosterol is the main contributor to the 1602 cm^{-1} band.[28]

The spectral features shown in Figure 2 appear to be quite similar at all measurement temperatures. Note that the shapes of the phospholipid Raman bands did not change from well-formed peaks to diffuse, broadened profiles at high temperature; this suggests that the double-membrane structure remained intact[24] and the cells were alive, albeit presumably not very active.

Intriguingly, though, the 1602 cm^{-1} band alone exhibits a marked decrease in intensity at elevated temperatures of 35.5, 36.8, and 38.3 °C. To illustrate this unique temperature de-

pendence better, we plot the ratios of the averaged peak areas of the four Raman bands at 1602, 1655, 1300, and 1003 cm^{-1} to that of the 1440 cm^{-1} band as a function of measurement temperature (Figure 3). A detailed description of peak area determination including baseline correction can be found in the Experimental Section. For the 1602 cm^{-1} band (mainly ergosterol), the peak area ratio remains unchanged between 26.1 and 34.5 °C, but at 35.5 °C it suddenly drops by as much as ≈47% and subsequently becomes constant again up to 38.3 °C (Figure 3A). In sharp contrast, the ratios for the bands at 1655 (mainly lipids, Figure 3B), 1300 (lipids, Figure 3C), and 1003 cm^{-1} (proteins, Figure 3D) show much less pronounced temperature dependence; they appear to be nearly independent of temperature with some fluctuation around the mean value. One might argue that the ratio for the 1655 cm^{-1} band also decreases slightly as the temperature is elevated. This decrease is estimated to be at most 19%, however, and is much smaller than the temperature response that we observed for the 1602 cm^{-1} band (19 vs. 47%). We can therefore conclude that the ergosterol band at 1602 cm^{-1} represents a highly sensitive indicator of a cell's surrounding temperature.

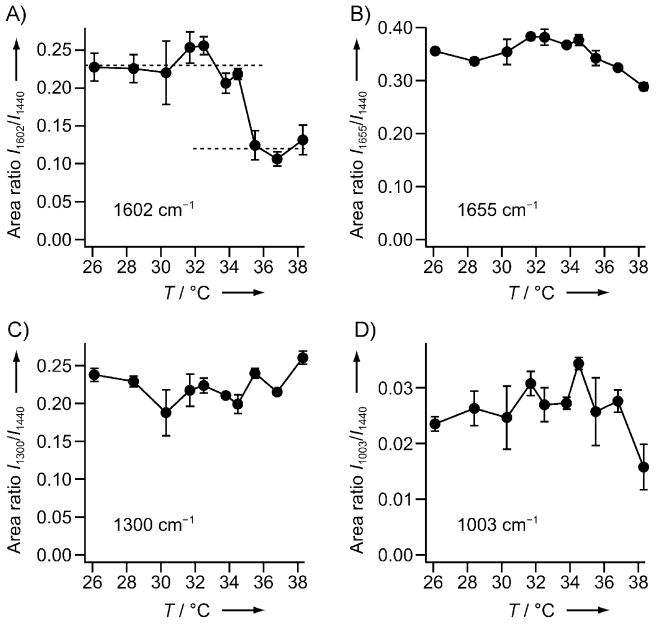

Figure 3. Temperature dependence of the peak area ratio of the A) 1602, B) 1655, C) 1300, and D) 1003 cm^{-1} bands to the 1440 cm^{-1} band. The peak area of each Raman band was derived by calculating the area between the band contour and a straight line connecting the two edges of the band (see the Experimental Section). Data represent mean values ± standard deviations of triplicate experiments.

The data presented in Figure 2 and 3 are averages over 30 cells, and as such they lack information on the statistical distributions of the band areas for individual cells. To examine whether the unique temperature response of the 1602 cm^{-1} band is also evident without averaging and normalization, the distributions of the Raman peak areas of the 1602 and 1440 cm^{-1} bands of all 30 cells are compared at 26.1 and 38.3 °C (Figure 4). The distribution of the 1602 cm^{-1} band area

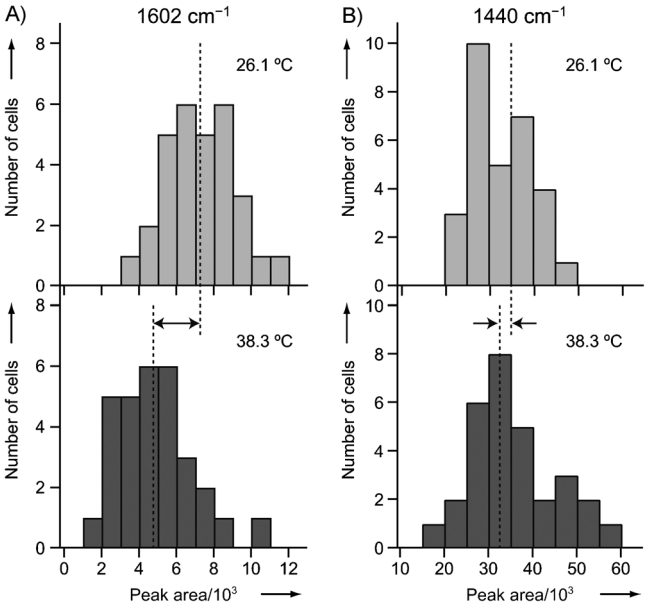

Figure 4. Peak area distributions of A) the 1602 cm^{-1}, and B) the 1440 cm^{-1} bands for yeast cells cultured at 26.1 °C (top) and 38.3 °C (bottom). Dashed lines indicate the mean values ($n = 30$).

at 38.3 °C is clearly shifted (by 2515 arbitrary units) toward lower value relative to that at 26.1 °C, whereas the area distribution of the 1440 cm^{-1} band does not change much between the two temperatures. Similar results were obtained from comparison of the 1602 cm^{-1} band with the other Raman bands; this confirms that the temperature response of the 1602 cm^{-1} band is significantly different from those of the other phospholipid/protein Raman bands at 1440, 1655, 1300, or 1003 cm^{-1}. In addition, it supports the assignment of the 1602 cm^{-1} band to ergosterol,[28] which is neither a phospholipid nor a protein.

This finding is consistent with a previous study by Shimizu and Katsuki[29] that showed that ergosterol biosynthesis in aerated budding yeast at 40 °C is lowered to only 32–35% relative to that at lower temperature (20 or 30 °C). This decrease was interpreted as a consequence of repression of the enzymes involved in the synthesis of mevalonate from acetyl-CoA, the initial stages of ergosterol biosynthesis.[14] We note, however, that Shimizu's and Katsuki's results were obtained by biochemical methods including extraction. Our study shows that the temperature sensitivity of the biosynthesis and metabolic activity in cells can now be probed in a nondestructive manner by Raman microspectroscopy.

Conclusions

We have revealed that the Raman band of ergosterol appearing at 1602 cm^{-1} in fission yeast cells responds to environmental temperature in such a sensitive way that the peak area abruptly decreases by ≈47% above 35 °C. This high sensitivity to temperature contrasts with less characteristic temperature dependence exhibited by other lipid and protein bands. It should be noted that this study, unlike the previous fluorescence studies,[5–11] is not an attempt to perform real-time moni-

toring of intracellular temperature. Rather it has shed new light on molecular-level understanding of the responses of intracellular components in living cells to environmental temperature, which is as central to chemical biology as intracellular thermometry.

Ergosterol represents an important distinction between fungi (including yeast) and animals (which use cholesterol instead of ergosterol). Indeed, many antifungal agents function by interacting with ergosterol. Amphotericin B, a well-known antifungal drug, for example, has been shown to kill yeast by directly binding ergosterol.[30] Our Raman microspectroscopic approach to monitoring ergosterol concentrations through the 1602 cm^{-1} band could help understanding, both in vivo and at the molecular level, of how these drugs work inside the cell and whether their antifungal ability is enhanced or impaired by changing environmental temperature.

Experimental Section

Cell culture: Fission yeast cells were cultured in YM broth (Acumedia, 7363) in a shaking incubator at 150 rpm at 10 different temperatures (26.1, 28.4, 30.3, 31.7, 32.5, 33.8, 34.5, 35.5, 36.8, and 38.3 °C) for 40 h so that cells reached stationary phase (see Figure 1 A). About 200 μL of the culture medium containing yeast cells was transferred to a glass-bottomed dish coated with poly-D-lysine (MatTek, 35G-1.5-14-C). Medium taken from the YM broth (1 mL) was centrifuged at 1200 rpm for 1 min (Beckman Coulter, Microfuge 16), and the supernatant fluid was then used to dilute the sample put into the poly-D-lysine-coated dish in order to prevent possible phase change and laser trapping of multiple cells. The diluted sample was used for Raman measurements. The growth curves of yeast cells (Figure 1 A) were determined by using a cell counting chamber (Marienfeld, 0640130) under the microscope.

Raman microspectroscopy: Raman spectroscopic measurements were performed with a laboratory-built confocal Raman microspectrometer, which has been described in detail elsewhere.[15–17] The 632.8 nm output of a He-Ne laser was used as the excitation light. The beam was magnified by a factor of ≈2.7 to effectively cover the exit pupil of the objective used. The expanded beam was introduced into an inverted microscope (Nikon, TE-2000 modified) and was focused onto the sample with an oil-immersion objective (CFI Plan Fluor, 100×, NA 1.3). The focus spot size was ≈1 μm. Backscattered Raman light was analyzed with an imaging spectrometer (HORIBA Scientific, iHR320) and detected with a back-illuminated, deep-depletion, liquid-N_2-cooled charge-coupled device (CCD) detector (Princeton Instruments, Spec-10:100) with 100×1340 pixels operating at −120 °C. A grating with 600 grooves per mm was used to cover a wide spectral range (> 2000 cm^{-1}) with an effective spectral resolution of 7 cm^{-1}. For bright-field observation, the sample was illuminated with a halogen lamp, and optical micrographs were acquired with a digital camera (Nikon, DS-Ri1) mounted on the microscope.

During the measurement, the sample temperature was kept the same as the culture temperature by use of an incubator (Tokai Hit, INU-ONICS-F1) mounted on the inverted-microscope stage. The target temperature was achieved with an accuracy of ±0.1 K by independently controlling the temperatures of four heaters available in the incubator and was monitored by measuring the temperature of water in the glass-bottomed dish with a thermometer. Fresh air (≈0.2 bar) was continuously passed into the stage-top incubator. At every temperature, Raman spectra of 30 (or more) randomly chosen yeast cells were obtained with 2.6 mW laser power at the sample point and with a 60 s exposure time. The relatively long exposure time yielded decent Raman spectra (see Figure 2), so no noise reduction of the recorded spectra, such as use of singular value decomposition,[15] was performed. Estimated spatial resolution was 0.3 μm in the lateral (*XY*) direction and 2.4 μm in the axial (*Z*) direction. Because the thickness of a fission yeast cell is typically ≈2 μm, the focal volume contained the entire cell along the *Z* direction.

Data analysis: Raman peak area was determined by integrating the area between the band contour and a baseline connecting the two edges of the band. The spectral interval used for the peak area determination was 1631–1686 cm^{-1} for the 1655 cm^{-1} band, 1587–1620 cm^{-1} for the 1602 cm^{-1} band, 1411–1495 cm^{-1} for the 1440 cm^{-1} band, 1279–1332 cm^{-1} for the 1300 cm^{-1} band, and 997–1008 cm^{-1} for the 1003 cm^{-1} band. Small variations in these intervals (± a few wavenumbers) had little effect on the peak area ratio shown in Figure 3. All spectral data analysis was performed with IGOR Pro 6 (Wave Metrics).

Acknowledgements

We thank Kung-Hui He for help in data analysis. This work was supported by the National Science Council of Taiwan (Grant NSC100-2113M-009-009-MY2).

Keywords: biophysics · ergosterol · fungi · lipids · Raman spectroscopy · temperature sensitivity

[1] L. H. Hartwell, J. Culotti, B. Reid, *Proc. Natl. Acad. Sci. USA* **1970**, *66*, 352.
[2] K. Ito, M. Wittekind, M. Nomura, K. Shiba, T. Yura, A. Miura, H. Nashimoto, *Cell* **1983**, *32*, 789.
[3] D. Michalovitz, O. Halevy, M. Oren, *Cell* **1990**, *62*, 671.
[4] P. Novick, R. Schekman, *Proc. Natl. Acad. Sci. USA* **1979**, *76*, 1858.
[5] L. H. Fischer, G. S. Harms, O. S. Wolfbeis, *Angew. Chem.* **2011**, *123*, 4640; *Angew. Chem. Int. Ed.* **2011**, *50*, 4546.
[6] F. Vetrone, R. Naccache, A. Zamarrón, A. Juarranz de La Fuente, F. Sanz-Rodríguez, L. M. Maestro, E. Martín Rodriguez, D. Jaque, J. García Solé, J. A. Capobianco, *ACS Nano* **2010**, *4*, 3254.
[7] J.-M. Yang, H. Yang, L. Lin, *ACS Nano* **2011**, *5*, 5067.
[8] C.-Y. Chen, C.-T. Chen, *Chem. Commun.* **2011**, *47*, 994.
[9] K. Okabe, N. Inada, C. Gota, Y. Harada, T. Funatsu, S. Uchiyama, *Nat. Commun.* **2012**, *3*, 705.
[10] K. Oyama, M. Takabayashi, Y. Takei, S. Arai, S. Takeoka, S. Ishiwata, M. Suzuki, *Lab Chip* **2012**, *12*, 1591.
[11] F. Ye, C. Wu, Y. Jin, Y.-H. Chan, X. Zhang, D. T. Chiu, *J. Am. Chem. Soc.* **2011**, *133*, 8146.
[12] J. Himms-Hagen, *Annu. Rev. Physiol.* **1976**, *38*, 315.
[13] M. Suzuki, V. Tseeb, K. Oyama, S. Ishiwata, *Biophys. J.* **2007**, *92*, L46.
[14] L. W. Parks, W. M. Casey, *Annu. Rev. Microbiol.* **1995**, *49*, 95.
[15] C.-K. Huang, M. Ando, H. Hamaguchi, S. Shigeto, *Anal. Chem.* **2012**, *84*, 5661.
[16] C.-K. Huang, H. Hamaguchi, S. Shigeto, *Chem. Commun.* **2011**, *47*, 9423.
[17] H. N. Noothalapati Venkata, S. Shigeto, *Chem. Biol.* **2012**, *19*, 1373.
[18] F. Sherman, *J. Cell. Comp. Physiol.* **1959**, *54*, 29.
[19] D. G. Grier, *Nature* **2003**, *424*, 810.
[20] G. P. Singh, C. M. Creely, G. Volpe, H. Grötsch, D. Petrov, *Anal. Chem.* **2005**, *77*, 2564.
[21] C. Xie, M. A. Dinno, Y.-q. Li, *Opt. Lett.* **2002**, *27*, 249.
[22] C. Xie, Y.-q. Li, *J. Appl. Phys.* **2003**, *93*, 2982.
[23] P. R. Carey, *Biochemical Applications of Raman and Resonance Raman Spectroscopies*, Academic Press, New York, **1982**.

[24] Y.-S. Huang, T. Karashima, M. Yamamoto, H. Hamaguchi, *Biochemistry* **2005**, *44*, 10009.
[25] D. Naumann, *Appl. Spectrosc. Rev.* **2001**, *36*, 239.
[26] L.-d. Chiu, M. Ando, H. Hamaguchi, *J. Raman Spectrosc.* **2010**, *41*, 2.
[27] Y.-S. Huang, T. Nakatsuka, H. Hamaguchi, *Appl. Spectrosc.* **2007**, *61*, 1290.
[28] L.-d. Chiu, F. Hullin-Matsuda, T. Kobayashi, H. Torii, H. Hamaguchi, *J. Biophotonics* **2012**, *5*, 724.
[29] I. Shimizu, H. Katsuki, *J. Biochem.* **1975**, *77*, 1023.
[30] K. C. Gray, D. S. Palacios, I. Dailey, M. M. Endo, B. E. Uno, B. C. Wilcock, M. D. Burke, *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 2234.

Received: February 25, 2013
Published online on April 29, 2013