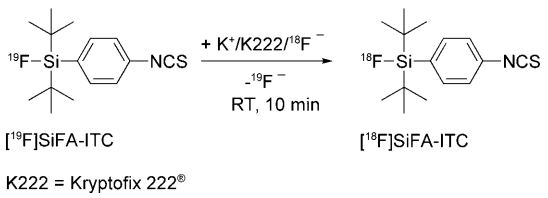
DOI: 10.1002/cbic.200900132

[^{18}F]SiFA-isothiocyanate: A New Highly Effective Radioactive Labeling Agent for Lysine-Containing Proteins

Pedro Rosa-Neto,[b] Björn Wängler,[c] Ljuba Iovkova,[d] Guido Boening,[c] Andrew Reader,[a] Klaus Jurkschat,[d] and Esther Schirrmacher*[a]

The design of targeted pharmaceuticals is booming, with antibodies or proteins as the most rapidly growing category.[1] In vivo imaging devices play an important role for their development and, although techniques based on fluorescence detection face some difficulties, the ease of fluorescent tag introduction into proteins is unsurpassed. Fluorescein isothiocyanate (FITC) is the most commonly used fluorescent labeling agent due to its easy reaction with lysine residues. Currently, there is no radioactive counterpart to FITC with a similar ease of introduction available for the radioactive labeling of proteins for in vivo imaging purposes. Positron emission tomography (PET) is an extremely powerful tool for in vivo imaging based on radioactivity detection, and has been used in the diagnosis of oncological and neurological diseases for many years.[2–4] Increasingly, its usefulness in drug development has been the focus of several studies.[1,5–7] The main setback of this technology is the complex procedure needed to introduce the positron emitter ^{18}F into proteins. With the advent of high-resolution scanners such as the High Resolution Research Tomograph (HRRT, Siemens), the optimal characteristics of ^{18}F (including its low positron energy, which results in images of high spatial resolution) have once more confirmed the important role this nuclide plays in PET imaging.[8] All ^{18}F-labeling procedures for proteins published so far are multistep procedures with low overall radiochemical yields and expensive equipment needs.[9,10] Following current standard procedures, ^{18}F is (after producing a water-free, highly nucleophilic [^{18}F]F^-/Kryptofix 222®/K^+ complex) incorporated into a molecule through a direct nucleophilic substitution on an alkyltosylate, aryltrimethylammonium ion or nitroaromatic; this generally requires high reaction temperatures and organic solvents to proceed efficiently.[11] These harsh conditions are unsuitable for proteins, which would be denatured under these conditions. Thus, the ^{18}F-labeling procedure of proteins has usually been a two- or multistep process with ^{18}F introduction into a small secondary labeling precursor under harsh conditions and subsequent reaction of these prosthetic groups with the protein of choice under mild, aqueous conditions. In the majority of cases, the protein-reacting groups of these secondary labeling precursors target either thiol moieties through a maleimide group or amino functions through acylation or alkylation reactions (for a review see ref. [9]). Unfortunately, most of the protein-reacting functionalities of these precursors decompose under the harsh conditions of the nucleophilic introduction of ^{18}F into the molecule. An isothiocyanide moiety would hardly survive the elevated temperatures and basic conditions usually applied for ^{18}F introduction. Consequently, they have to be either protected or else generated only after the ^{18}F has been introduced into the secondary labeling precursor.[12] Even the most recent, improved methods for ^{18}F introduction into proteins suffer from low preparative radiochemical yields (usually in the range of 1–5%) and long reaction times (90–120 min).[13–15]

Recently, new approaches to radiolabeling involving silicon-fluorine chemistry have been reported.[16–18] Our group introduced a completely new labeling method based on an organosilicon containing fluoride acceptor SiFA (**Si**licon **F**luoride **A**cceptor) that is based on an isotopic ^{18}F for ^{19}F-exchange at a silicon atom.[17,18] The SiFA method was shown to offer a quick and efficient introduction of ^{18}F into various peptides in both one- two-step reactions. Based on the convenient widely used introduction of fluorescent tags into proteins through an isothiocyanate-containing dye,[19] we decided to investigate the protein-labeling potential of [^{18}F]SiFA-isothiocyanate ([^{18}F]SiFA–ITC, Scheme 1), which would primarily target lysine residues. Isothiocyanates react with primary amino functions to form stable thioureas (Scheme 2). We chose three proteins of different sizes (rat serum albumin (RSA, 66 kDa), apotransferrin (76–81 kDa) and bovine IgG (144 kDa)) as sample proteins for radiolabeling.



Scheme 1. Isotopic exchange reaction of [^{19}F]SiFA-isothiocyanate (3–3.6 μg; 10–12 nmol) in DMSO to form the secondary labeling precursor [^{18}F]SiFA-isothiocyanate within 10 min at room temperature with 95% radiochemical yields.

[a] *Dr. A. Reader, Dr. E. Schirrmacher*
Montreal Neurological Institute, McGill University
3801 rue University, Montreal, H3A 2B4 (Canada)
Fax: (+1) 514-398-8195
E-mail: esther.schirrmacher@mcgill.ca

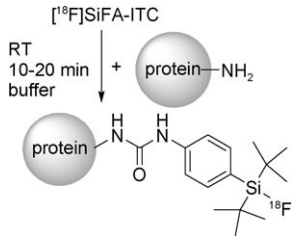
[b] *Dr. P. Rosa-Neto*
Montreal Neurological Institute, McGill University, Douglas Research Institute
Montreal, H3A 2B4 (Canada)

[c] *Dr. B. Wängler, Dr. G. Boening*
Department of Nuclear Medicine, Ludwig-Maximillians-Universität
Marchionstrasse 15, 81377 Munich (Germany)

[d] *L. Iovkova,[+] Prof. Dr. K. Jurkschat[+]*
Inorganic Chemistry, TU Dortmund
Otto-Hahn-Strasse 6, 44227 Dortmund (Germany)

[+] *L.I. and K.J. synthesized the SiFA–ITC.*

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.200900132.



Scheme 2. Conjugation of crude [^{18}F]SiFA–ITC (10–12 nmol, 2–3 GBq) with protein in buffer at pH 9.0 at room temperature with yields of 30–80% for the tested RSA, apotransferrin and bovine IgG.

The efficacy of the synthesis of [^{18}F]SiFA–ITC by isotopic exchange under very mild conditions depended on the precursor concentration ([^{19}F]SiFA–ITC) and on the basicity of the reaction mixture. With increasing concentrations of the carbonate containing basic [^{18}F]F$^-$/Kryptofix 222®/K$^+$ complex, decomposition of the Si–F bond was observed; this decomposition generated unbound ^{18}F$^-$. Thus, the less basic potassium oxalate system was used instead of the more common carbonate system. Generally, the dried ^{18}F$^-$ (37–74 GBq) was dissolved in 900 μL DMSO and used as a stock solution. For each 20–60 μL of this solution (about 2–3 GBq), 10–12 nmol of [^{19}F]SiFA–ITC were needed to obtain optimal radiochemical yields (>90%). The calculated specific activities of [^{18}F]SiFA–ITC were 100-160 GBq μmol^{-1} (2700–4500 Ci mmol^{-1}). Although these specific activities are by all means in a most acceptable range for human use in PET, the achievement of higher specific activities is always preferable. The syntheses of Si[^{18}F]-containing building blocks from SiOH precursors has effectively proven this.[16]

The conjugation of the secondary labeling precursor [^{18}F]SiFA–ITC to the protein depends on 1) the pH, 2) the lysine content of the protein, and 3) the protein concentration. We investigated different reaction buffers between pH 8.0 and 9.5. Higher pH-values lead to a faster reaction of the isothiocyanate with the protein, but also to a faster hydrolysis of the Si–F bond, which is detected as free [^{18}F]fluoride in the reaction mixture. At pH <9.0, the reaction proceeded too slowly for the short-lived radioisotope. At pH 9.0 we achieved the highest conjugation yields for the RSA protein within 10 min. The apotransferrin and the bovine IgG reacted more slowly, possibly due to the presence of fewer lysine residues in the proteins, or to less accessible ones. The maximal conjugation yields of 30–45% for those two proteins were obtained after 10–20 min (Table 1). Hydrolysis of the Si–F bond was considerably slower (after 30–40 min) at pH 9.0. Conjugation yields of up to 80% were achieved for RSA because of its high lysine content (0.89 Lys/kDa versus 0.71 Lys/kDa for apotransferrin and 0.38 Lys/kDa for bovine IgG). The amount of [^{19}F]SiFA–ITC used in the reaction dictates the protein concentration because a ratio of 100:1 ([$^{18/19}$F]SiFA–ITC/protein) will most likely result in significant changes of the nature of the protein due to over-conjugation. We therefore investigated protein concentrations corresponding to a range of 10:1, 2:1, 1:1 and 1:6 (1:8 for Bovine IgG) ratio of [^{19}F]SiFA–ITC/protein and determined the radiochemical yields for those ratios only (Table 1). The influence of the SiFA–ITC to protein ratio to the RCY of the labeled protein is outlined in Figure 1. The highest radiochemical yields (RCYs) for all investigated proteins are obtained in the presence of a high protein/SiFA ratio.

Table 1. Reaction parameters for the ^{18}F labeling of proteins with [^{18}F]SiFA–ITC.

Protein entry[a]	SiFA–ITC μg [nmol][a]	Protein μg [nmol]	SiFA/protein ratio[b]	Labelling yields[c] (%)
1. RSA	3 [10]	3840 [60]	1:6	80
2. RSA	0.9 [3]	20 [0.3]	10:1	30
3. RSA	3.6 [12]	800 [12]	1:1	60
4. RSA	3 [10]	334 [5]	2:1[d]	55
5. apotransferrin	1.5 [5]	2300 [30]	1:6	45
6. apotransferrin	0.9 [3]	23 [0.3]	10:1	30
7. apotransferrin	3.6 [12]	920 [12]	1:1	30
8. apotransferrin	3 [10]	384 [5]	2:1	40
9. bovine IgG	0.75 [2.5]	2500 [20]	1:8	40
10. bovine IgG	0.9 [3]	50 [0.3]	10:1	30
11. bovine IgG	3.6 [12]	1000 [12]	1:1	40
12. bovine IgG	3 [10]	417 [5]	2:1	35

[a] From a stock solution of [^{18}F]SiFA–ITC (2–3 GBq, 3–3.6 μg, 10–12 nmol in 30–60 μL DMSO). [b] Reactions were done in DMSO/carbonate buffer (pH 9) mixtures (crude [^{18}F]SiFA–ITC (2–3 GBq, 0.9–3.6 μg, 3–12 nmol in 30–120 μL DMSO) + protein (0.3–60 nmol in 25 μL carbonate buffer (pH 9)) + carbonate buffer (pH 9) (150–700 μL)). [c] Reaction time was between 10–20 min and conjugation yields refer to HPLC chromatograms of the crude reaction mixture (HPLC conditions see the Supporting Information), N = 10–15, error margins for RCYs were between 3–5% [d] these labeling conditions have been used for conducting the animal experiments.

The purification of the final radiolabeled proteins was achieved by either HPLC or centrifugal filters. While HPLC purifications take about 15-20 min, depending on the retention times

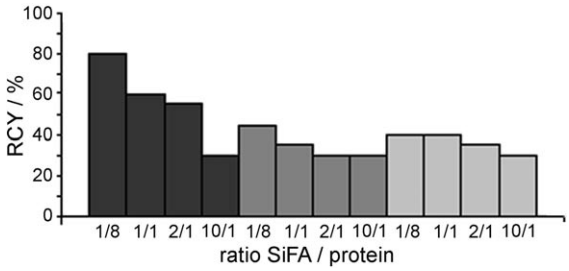


Figure 1. Conjugation of crude [^{18}F]SiFA–ITC (3–12 nmol SiFA–ITC, 2–3 GBq [^{18}F]F/K222®/oxalate/K$^+$) to RSA (dark gray), Appotransferrin (gray) and Bovine IgG (light gray) using different ratios of [^{18}F]SiFA–ITC to protein in DMSO/carbonate buffer (pH 9) (N = 10–15, columns represent mean values with error margins of 3–5% for RCYs; for specific amounts of SiFA–ITC and proteins see Table 1, RCY = radiochemical yield).

of the ^{18}F-labeled proteins, the radiochemical purity of the labeled proteins was $>99\%$. With the centrifugal filters, purifications could be performed generally within 12 min and the radiochemical purity of the product was $>95\%$ but it was not always reproducible. With increasing amounts of proteins and increasing molecular weights of proteins, the centrifugal filter purification worked less efficiently, probably due to clogging of the filter membrane; this reduced the radiochemical purity of the ^{18}F-labeled proteins to $<90\%$. We also used NAP-5 columns (Sephadex G-25 DNA grade, GE Healthcare) for the purification of the [^{18}F]SiFA-labeled proteins, but were not able to achieve consistent radiochemical purities of $>95\%$, due to occasional smearing of the [^{18}F]SiFA–ITC across the NAP column contaminating the protein fraction; this might be due to slight differences of the product yields in the reaction mixtures. For a reliably high purity of radiolabeled protein without optimization, HPLC purification was superior. The yielded [^{18}F]SiFA-protein conjugates had a shelf-life of more than 1 hour in isotonic saline and displayed only negligible decomposition. To obtain preliminary data about the stability of the [^{18}F]SiFA-labeled RSA (ratio [^{18}F]SiFA–ITC/RSA 2:1, protein entry 4, Table 1) we added 5 MBq of the HPLC-purified compound (in 10 μL of DMSO) to 1 mL of freshly prepared rat serum at 37 °C and drew aliquots after 30, 60 and 120 min. These aliquots were investigated by size exclusion HPLC. After 120 min approximately 10–15% of the detected radioactivity accounted for smaller compounds; this proved that most of the [^{18}F]SiFA–RSA was still intact. These experiments justified the following in vivo study using animal PET.

To prove the metabolic stability of the ^{18}F label in vivo, we investigated the radioactivity distribution of [^{18}F]SiFA-RSA (ratio [^{18}F]SiFA–ITC/RSA 2:1, cf. protein entry 4, Table 1) in vivo in a rat. RSA is mainly metabolized in the liver and the uptake of any radioactivity in blood vessels and liver would therefore be expected. The pronounced liver uptake of the SiFA-labeled RSA is probably a result of the higher lipophilicity imposed by the SiFA derivatisation (Figure 2). In our previous publications we found a less pronounced liver uptake when differently SiFA-labeled RSA was used in vivo in rats.[20,21] This suggests that the position of SiFA labeling in RSA determines the metabolic fate to a certain extent. Radioactivity uptake into the bone structure would suggest free, unbound $^{18}F^-$. Radioactivity uptake into the bladder can either be caused by free fluoride, or by hydrophilic metabolites of the ^{18}F protein. The obtained PET images suggested very low rates of defluorination of the ^{18}F-silicon thiourea during the first 60–90 min post injection (Figure 2 and also time–activity curves in the Supporting Information). Slightly more evident images of the rat's bone structure observed in the 110–120 min time frames suggest that enzymatic steps responsible for the defluorination occur late in the the^{18}F-silicon thiourea metabolic pathways and do not compromise image quality. Blood samples drawn at several time points after injection were analyzed with radio-HPLC to further determine metabolic stability. No peak corresponding to [^{18}F]SiFA–ITC and only minor amounts (10–12%) of compounds of low molecular weight were detected, indicating the metabolic stability of the thiourea bond. However in the very late blood samples (160 min p.i.) 65% of the detected radioactivity could be assigned to small unidentified ^{18}F-labeled compounds (Supporting Information). PET results may be partially biased by the anesthesia with isofluorane.

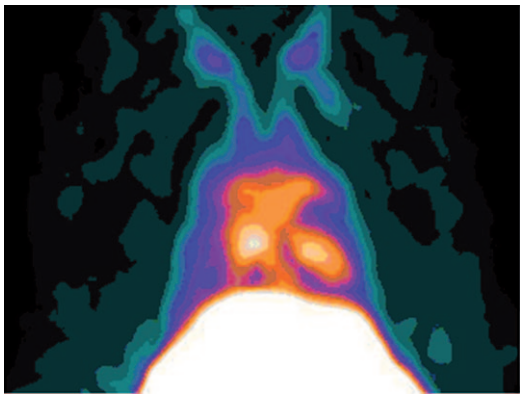


Figure 2. PET images obtained from a male Sprague Dawley rat after the injection of [^{18}F]SiFA-RSA. Maximum intensity projection (MIP) after injection of 7.4 MBq (0.2 mCi) of [^{18}F]SiFA-RSA in a healthy rat. Data were acquired 5 s post tracer administration over 150 s with the Concorde MicroPET R4 small animal tomograph and reconstructed on a Linux cluster (Transtec AG, Tübingen, Germany) using 3D OSEM (ordered subsets expectation maximization, 2 iterations) followed by 3D OP-MAP (ordinary Poisson maximum a posteriori, 18 iterations with beta 0.05) in 256×256×63 voxels (0.4×0.4×1.2 mm). To reduce image noise, a post reconstruction 3D Gaussian filter with 3 mm full width at half maximum (FWHM) was applied. Tracer accumulation was clearly observed in the great arteries, the liver and the left ventricle of the heart. Furthermore the right ventricle and atria were noticeable.

In conclusion, we have shown that [^{18}F]SiFA-isothiocyanate is a versatile secondary labeling precursor for the ^{18}F introduction into proteins. The preparative radiochemical yields of labeled proteins are high enough (20–40% non-decay corrected within 40–50 min) to allow centers without their own cyclotron facility to produce ^{18}F-labeled proteins in sufficient amounts for in vivo imaging. Reaction times of 40–50 min including purification present a marked improvement over existing methods. Moreover, we have shown that the label is remarkably stable under in vivo conditions and particularly suitable for extended PET imaging protocols. [^{18}F]SiFA–ITC is an applicable radioactive analogue to FITC, allowing the reliable and convenient introduction of ^{18}F into proteins containing lysine residues.

Experimental Section

The syntheses and analytical data, HPLC chromatograms as well as the pertinent information about the in vivo PET scan can be found in the Supporting Information.

Animal experiments were approved by the Animal Ethics Committee of the Lady Davis Institute for Medical Reseach, McGill University and abided by the guidelines of the Canadian Council of Animal Care.

Radiochemistry

[^{18}F]SiFA-isothiocyanate ([^{18}F]SiFA–ITC): [^{18}F]Fluoride in $H_2{}^{18}O$ (37–74 GBq) from the cyclotron was passed over a QMA cartridge preactivated with sodium carbonate solution (5 mL) and washed with

water (10 mL). The $^{18}F^-$ was eluted with a solution of Kryptofix 222® (10 mg, 27.2 μmol), sodium oxalate solution (0.5 M, 5 μL, 2.5 μmol, 335 μg), water (50 μL) and acetonitrile (800 μL). The solvents were evaporated at 90 °C under a stream of nitrogen and under reduced pressure (700 mbar). The azeotropic drying was repeated twice with the addition of 1 mL acetonitrile respectively. After the third evaporation, the pressure was reduced to 10 mbar for 1 min to ensure the complete absence of water. The remaining $[^{18}F]F^-$/Kryptofix 222®/K^+ complex was dissolved in DMSO (900 μL) and used as a stock solution for experiments.

The dried $[^{18}F]F^-$/Kryptofix 222®/K^+ complex (2–3 GBq) in DMSO (20–60 μL) was added to SiFA–ITC (3–3.6 μg; 10–12 nmol; from stock solution in 10–60 μL DMSO) and allowed to react for 10 min at room temperature without stirring. Radiochemical yields were determined with radio-TLC (stationary phase: Si-60 SiO_2 plates) in neat ethyl acetate ($R_f = 0.7$) and with HPLC ($t_R = 19.5$ min with a flow of 0.4 mL min^{-1}; Supporting Information). The crude reaction mixture was used without purification for subsequent protein labeling.

^{18}F-labeled protein: A solution of the protein (0.3–60 nmol, Table 1) in carbonate buffer (0.1 M, pH 9.0, 25 μL) plus additional carbonate buffer (0.1 M, pH 9.0; 150–700 μL) was added to the crude reaction mixture of $[^{18}F]$SiFA–ITC (1–10 equiv SiFA–ITC, 2–3 GBq) in DMSO (30–120 μL) and left at room temperature for 10–20 min. The solution was then transferred to a centrifugal filter (Vivaspin Sartorius, VWR, Canada) and centrifuged at 14000 *g* for 3 min. Phosphate buffered saline (200 μL, pH 7.4) was added three times between repetitions of the centrifugation step. The resulting solution of the $[^{18}F]$SiFA-labeled protein was used directly for in vivo studies. Alternatively, the reaction mixture was injected into the HPLC system (Supporting Information) and the peak at the appropriate retention time (15–15.2 min) was collected and diluted with isotone saline. For the animal experiments, a SiFA/RSA ratio of 2:1 was used for injection (Protein entry 4, Table 1).

Acknowledgements

The authors wish to acknowledge the cyclotron personnel of the MNI for their generous supply of ^{18}F, M. Slim for helping with radiolabeling and Antonio Aliaga for his help with the PET scan.

Keywords: fluorine · imaging agents · positron emission tomography · protein labeling · radiochemistry

[1] G. A. M. S. van Dongen, G. W. M. Visser, M. N. Lub-de Hooge, E. G. de Vries, L. R. Perk, *Oncologist* **2007**, *12*, 1379–1389.
[2] C. K. Hoh, *Nucl. Med. Biol.* **2007**, *34*, 737–742.
[3] A. M. Groves, T. Win, S. B. Haim, P. J. Ell, *The Lancet Oncol.* **2007**, *8*, 822–830.
[4] J. C. Baron, G. L. Lenzi, T. Brucke, R. Frackowiak, W. D. Heiss, P. Pantano, O. B. Paulson, P. M. Rossini, J. Schoenen, P. J. Touboul, J. Vion-Dury, R. Von Kummer, *Eur. J. Neurol.* **1998**, *5*, 5–15.
[5] D. S. Boss, R. V. Olmos, M. Sinaasappel, J. H. Beijnen, J. H. M. Schellens, *Oncologist* **2008**, *13*, 25–38.
[6] P. McGuire, O. D. Howes, J. Stone, P. Fusar-Poli, *Trends Pharmacol. Sci.* **2008**, *29*, 91–98.
[7] R. J. Hargreaves, *Clin. Pharmacol. Ther.* **2008**, *83*, 349–353.
[8] H. W. A. M. Jong, F. H. P. Velden, R. W. Kloet, F. L. Buijs, R. Boellaard, A. A. Lammertsma, *Phys. Med. Biol.* **2007**, *52*, 1505–1526.
[9] R. Schirrmacher, C. Waengler, E. Schirrmacher, *Mini-Rev. Org. Chem.* **2007**, *4*, 317–329.
[10] S. M. Ametamey, M. Honer, P. A. Schubiger, *Chem. Rev.* **2008**, *108*, 1501–1516.
[11] S. E. Snyder, M. R. Kilbourne, *Chemistry of Fluorine-18 Radiopharmaceuticals* (Ed.: M. J. Welch, C. S. Redvanly), Wiley, Chichester, **2004**, pp. 195–227.
[12] G. Vaidyanathan, M. R. Zalutsky, *Nucl. Med. Biol.* **1995**, *22*, 759–764.
[13] R. R. Flavell, P. Kothari, M. Bar-Dagan, M. Synan, S. Vallabhajosula, J. M. Friedman, T. W. Muir, G. Ceccarini, *J. Am. Chem. Soc.* **2008**, *130*, 9106–9112.
[14] X. Li, J. M. Link, S. Stekhova, K. J. Yagle, C. Smith, K. A. Krohn, J. F. Tait, *Bioconjugate Chem.* **2008**, *19*, 1684–1688.
[15] F. Wuest, M. Berndt, R. Bergmann, J. van den Hoff, J. Pietzsch, *Bioconjugate Chem.* **2008**, *19*, 1202–1210.
[16] L. Mu, A. Höhne, P. A. Schubiger, S. M. Ametamey, K. Graham, J. E. Cyr, L. Dinkelborg, T. Stellfeld, A. Srinivasan, U. Voigtmann, U. Klar, *Angew. Chem.* **2008**, *120*, 5000–5003; *Angew. Chem. Int. Ed.* **2008**, *47*, 4922–4925.
[17] E. Schirrmacher, B. Waengler, M. Cypryk, G. Bradtmoller, M. Schafer, M. Eisenhut, K. Jurkschat, R. Schirrmacher, *Bioconjugate Chem.* **2007**, *18*, 2085–2089.
[18] R. Schirrmacher, G. Bradtmoller, E. Schirrmacher, O. Thews, J. Tillmanns, T. Siessmeier, H. G. Buchholz, P. Bartenstein, B. Waengler, C. M. Niemeyer, K. Jurkschat, *Angew. Chem.* **2006**, *118*, 6193–6197; *Angew. Chem. Int. Ed.* **2006**, *45*, 6047–6050.
[19] M. M. Akhavan, S. A. Ebrahimi, M. Mahmoudian, *J. Pharmacol. Toxicol. Methods* **2006**, *53*, 206–214.
[20] L. Iovkova, B. Waengler, E. Schirrmacher, R. Schirrmacher, G. Quandt, G. Boening, M. Schuermann, K. Jurkschat, *Chem. Eur. J.* **2009**, *15*, 2140–2147.
[21] B. Waengler, G. Quandt, L. Iovkova, E. Schirrmacher, C. Waengler, G. Boening, M. Hacker, M. Schmoeckel, K. Jurkschat, P. Bartenstein, R. Schirrmacher, *Bioconjugate Chem.* **2009**, *20*, 317–321.
[22] R. B. Weisenfeld, *J. Org.Chem.* **1986**, *51*, 2434–2436.

Received: March 11, 2009
Published online on May 6, 2009

