DOI: 10.1002/adsc.200700094

Efficient Whole-Cell Biocatalytic Synthesis of *N*-Acetyl-D-neuraminic Acid

Ping Xu,[a,b,*] Jian Hua Qiu,[a,e] Yi Nan Zhang,[a,e] Jing Chen,[a,e] Peng George Wang,[a] Bing Yan,[c] Jing Song,[a] Ri Mo Xi,[d] Zi Xin Deng,[b] and Cui Qing Ma[a,*]

[a] State Key Laboratory of Microbial Technology, Shandong University, Jinan 250100, Shandong Province, People's Republic of China
Fax: (+86)-531-8856-7250; e-mail: pingxu@sdu.edu.cn or macq@sdu.edu.cn
[b] Key Laboratory of Microbial Metabolism, Ministry of Education, College of Life Science & Biotechnology, Shanghai Jiao Tong University, Shanghai 200240, People's Republic of China
[c] School of Pharmaceutical Sciences, Shandong University, Jinan 250100, Shandong Province, People's Republic of China
[d] Department of Chemistry, Shandong University, Jinan 250100, Shandong Province, People's Republic of China
[e] J. H. Qiu, Y. N. Zhang and J. Chen contributed equally to this work.

Received: February 11, 2007

Supporting information for this article is available on the WWW under http://asc.wiley-vch.de/home/.

Abstract: *N*-Acetyl-D-neuraminic acid (Neu5Ac) was efficiently synthesized from lactate and a mixture of *N*-acetyl-D-glucosamine (GlcNAc) and *N*-acetyl-D-mannosamine (ManNAc) by whole cells. The biotransformation utilized *Escherichia coli* cells (Neu5Ac aldolase), *Pseudomonas stutzeri* cells (lactate oxidase components), GlcNAc/ManNAc and lactate. By this process, 18.32 ± 0.56 g/liter Neu5Ac were obtained from 65.61 ± 2.70 g/liter lactate as an initial substrate input. Neu5Ac (98.4 ± 0.4% purity, 80.87 ± 0.79% recovery yield) was purified by anionic exchange chromatography. Our results demonstrate that the reported Neu5Ac biosynthetic process can compare favorably with natural product extraction or chemical synthesis processes.

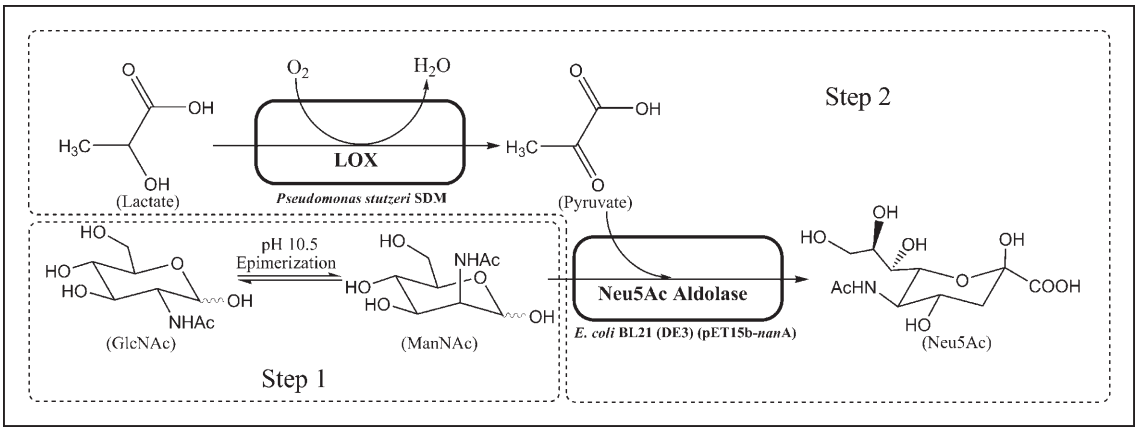
Keywords: *N*-acetyl-D-neuraminic acid (Neu5Ac); biotransformation; lactate oxidase component; Neu5Ac aldolase; sialic acids; whole cells

Sialic acids are derivatives of neuraminic acids and are present in higher animals and some microorganisms. They are often found at the terminal position of glycoproteins and glycolipids on the cell surface. Sialic acids are involved in several cell functions. For example, they represent recognition sites for various physiological receptors, such as selectins and siglecs.[1] *N*-Acetyl-D-neuraminic acid (Neu5Ac), *N*-glycolyl-D-neuraminic acid (Neu5Gc), and *N*-acetyl-9-*O*-acetyl-neuraminic acid (Neu5, $9Ac_2$) are the three most frequently occurring members of the sialic acid family. Among sialic acids, only Neu5Ac is ubiquitous, while the others are not found in all species. Neu5Ac can be prepared by hydrolysis of various natural products,[2] enzymatic conversion[3] or chemical synthesis.[4] Chemical synthesis of Neu5Ac is often restricted to low yield and unsatisfactory stereoselectivity, in addition to tedious protection and deprotection steps. Enzymatic synthesis of Neu5Ac from *N*-acetyl-D-mannosamine (ManNAc) and pyruvate using Neu5Ac aldolase (EC 4.1.3.3, NanA) as the catalyst has been reported.[3,5] However, preparation of the enzyme, which requires purification or partial purification, makes the process more complicated and costly.

In the past decades, biocatalysis using whole cells has emerged as an important tool for the large-scale synthesis of bulk chemicals, pharmaceutical and agrochemical intermediates, active pharmaceuticals, and food ingredients. The realistic large-scale synthesis routes are probably based on the combination of biocatalysis and chemocatalysis.[6]

In this work, we reported an efficient whole-cell biocatalysis procedure for Neu5Ac synthesis utilizing NanA and lactate oxidase component (LOX) as catalyzing enzymes and lactate and ManNAc/GlcNAc[3b,e] as substrates (Scheme 1).

Because the racemic and stereochemical pure forms of lactate are much cheaper than pyruvate (the price is about 17 times lower, calculated by the data from http://www.sigma-aldrich.com/), the lactate was used as a starting substrate instead of pyruvate in the reaction. In our previous work, we have described an efficient enzymatic route for synthesizing pyruvate from lactate using whole cells of *Acinetobacter* sp. or *Pseudomonas* sp. The reported LOX produced by these strains could effectively oxidize lactate into pyruvate.[7,8] Therefore the cells of *Pseudomonas stutzeri*



Scheme 1. Procedure for the production of Neu5Ac from GlcNAc/ManNAc and lactate. Step 1, preparation of ManNAc/GlcNAc by the alkaline-catalyzed epimerization. Step 2, production of Neu5 Ac by coupling whole-cell conversion of lactate and ManNAc.

SDM capable of efficiently producing LOX were used to supply pyruvate. From the results of control experiments (-○-, Control A in Figure 1), it was determined that strain SDM could not synthesize Neu5Ac at all, but had the ability to catalyze lactate to pyruvate. The maximum concentration of the accumulated pyruvate was 49.29 ± 2.38 g/liter that represents a yield of 83.11 % from lactate (Figure 1 b).

In order to produce NanA on a large scale, we have constructed a plasmid pET15b-*nan*A containing the gene of NanA that was cloned from the *E. coli* K-12 strain.[9] It is obviously shown that NanA was overexpressed in the host strain *E. coli* BL21 (DE3) with the pET system under the control of T7 promoter by adding isopropyl-β-D-thiogalactopyranoside (IPTG) (see Figure S1 in the Supporting Information). The NanA expression level in the *E. coli* BL21 (DE3) (pET15b-*nan*A) (13.51 U/mg protein) was about 56 times higher than that expressed in the original strain *E. coli* K-12 (0.24 U/mg protein). In Figure S1, the arrow indicates the NanA expressed in the recombinant *E. coli* strain. The protein molecular weight of NanA was 33 kDa consistent with a previous report.[9]

In the control experiments (-△-, Control B in Figure 1) strain *E. coli* BL21 (DE3) (pET15b-*nan*A) produced 3.69 ± 1.17 g/liter of Neu5Ac and 7.73 ± 0.52 g/liter of pyruvate in 40 hours. It was reported that the "normal" role of NanA was to cleave Neu5Ac into pyruvate and ManNAc, and an excess of pyruvate could push the equilibrium toward Neu5Ac.[3e] So strain SDM should be used in the reaction to supply enough pyruvate and shift the equilibrium to Neu5Ac.

We conducted the reaction as a 400-mL batch process in a 3,000-mL flask. At the beginning of the reaction, cells of *E. coli* BL21 (DE3) (pET15b-*nan*A) (2.1 g in wet weight, about 625 U NanA), cells of strain SDM (14.5 g in wet weight, about 727 U of LOX), 19.62 g ManNAc/GlcNAc mixture, and 26.25 g L-lactate were added in the shaking flask. The reaction kinetics were monitored (Figure 1). The concentration of Neu5Ac increased with time as the lactate and ManNAc concentrations decreased. At the maximum of Neu5Ac synthesis (20 h), the mixture comprised 18.32 ± 0.56 g/liter Neu5Ac, 35.15 ± 3.85 g/liter lactate, 24.46 ± 2.80 g/liter ManNAc, and 15.79 ± 3.27 g/liter pyruvate. The maximal concentration of pyruvate was 16.28 ± 3.01 g/liter, which was about 3 times lower than that in control A. This might be caused by the lower oxygen level in the reaction solution. We also detected acetic acid in the broth as evidence for the lack of oxygen. To maximize the production of Neu5Ac, we stopped the reaction at 20 h, when the concentration of Neu5Ac reached the maximum.

The final purification of Neu5Ac from the reaction mixture was relatively easy and inexpensive. Using anionic exchange chromatography purification and crystallization in glacial acetic acid, 5.93 ± 0.19 g Neu5Ac were obtained. The recovery of pure Neu5Ac from raw reaction mixture is 80.87 ± 0.79 % and its final purity is 98.4 ± 0.4 % as determined by HPLC/UV with detection at 205 nm. The purified Neu5Ac was characterized by ^{13}C NMR (see Figure S2 in the Supporting Information).

Neu5Ac can be used as a starting reactant for the enzymatic or chemical synthesis of biologically important derivatives and oligosaccharides. To date, little has been reported on the synthesis of Neu5Ac by whole cells.[10] By combining chemical synthesis and biocatalysis, we produced 18.32 g/liter of Neu5Ac from 223.18 g/liter of GlcNAc and 65.61 g/liter of lactate. The key component of this process is a biotransformation reaction that used whole cells containing NanA and LOX, respectively as the catalysts. We are currently testing a similar system containing LOX to

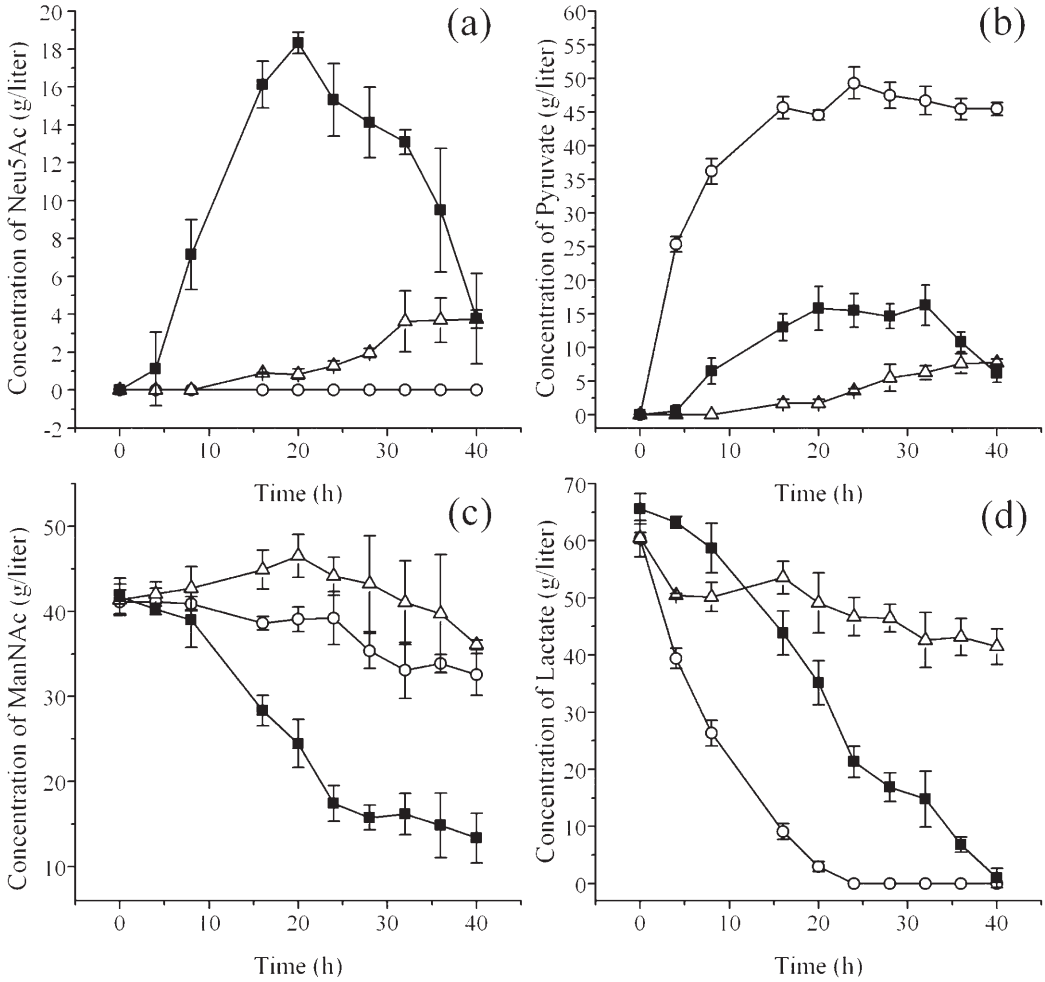


Figure 1. Time course for the formation of Neu5Ac from lactate and ManNAc/GlcNAc by whole cells. (-○-, Control A) *P. stutzeri* SDM with LOX only, (-△-, Control B) *E. coli* that over-expressed NanA only, (-■-) the recombinant of *E. coli* that over-expressed NanA and *P. stutzeri* SDM with LOX. (**a**), (**b**), (**c**) and (**d**) indicate the concentrations of Neu5Ac, pyruvate, ManNAc and lactate, respectively.

synthesize other compounds such as L-3,4-dihydroxyphenylalanine and L-phenylacetylcarbinol from lactate as a starting substrate.

In summary, the advantages of the reported whole-cell biocatalysis process are as follows: (a) the whole-cell catalytic process did not require preparation of the purified or partially purified enzymes; (b) a pyruvate supplying system catalyzed by LOX was used in the process so that the inexpensive substrate lactate could be used as a substitute for pyruvate; (c) the purification procedures of Neu5Ac were simple and efficient.

Experimental Section

General Remarks

Sodium L-lactate (≥80%) was obtained from Wujiang Ci Yun Fragrance Co., Ltd.; GlcNAc (≥97%) was supplied by Shandong Dongying Marine Bio-chemical Co., Ltd.; Neu5Ac (≥98%), ManNAc, GlcNAc, sodium pyruvate and sodium L-lactate (≥99%) were purchased from Sigma. Anionic Resin HZ-201 was obtained from Hua Zhen Scientific and Technological Co. Ltd.; IPTG was purchased from Merck Eurolab (Strasbourg, France); the protein weight marker was obtained from MBI fermentas.

Neu5Ac, ManNAc, GlcNAc, pyruvate and lactate were determined using HPLC (Agilent 1100 series, Hewlett-Packard), equipped with a Bio-Rad Aminex HPX-87H column (300×7.8 mm) with a mobile phase of 6 mM H_2SO_4. The flow rate was 0.5 mL min^{-1}, and the UV detector was set at 205 nm. The column temperature was maintained at 60 °C.[11]

NanA Cloning and Over-expression

The NanA gene (*nan*A) was amplified by PCR using *E. coli* K-12 (ATCC 47076) chromosomal DNA as a template,[12] and 5′-TTACCATGGATGGCAACGAATTTACG-3′ (*Nco*I cleavage site underlined) and 5′-ATAGGATCCT-

CACCCGCGCTCTTGCAT-3′ (*Bam*HI cleavage site underlined) as primers based on the published *nan*A gene sequence (GenBank accession no. D00067). The 0.9-kb PCR product was digested with *Nco*I and *Bam*HI and purified using Wizard® PCR Preps DNA Purification System kit (Promega).[3e,13] The purified fragment was inserted between the *Nco*I and *Bam*HI sites of pET15b (Novagen) to give pET15b-*nan*A. The constructed plasmid pET15b-*nan*A was transformed into *E. coli* BL21 (DE3) (Novagen). *E. coli* BL21 (DE3) (pET15b-*nan*A) was cultured in 300 mL of medium containing glucose 1%, $Na_2HPO_4{\cdot}12H_2O$ 0.6%, KH_2PO_4 0.3%, NaCl 0.05%, NH_4Cl 0.5%, $MgSO_4{\cdot}7H_2O$ 0.055%, yeast extract 0.2%, and 100 μg/mL ampicillin in a 1-liter flask at 250 rpm, 37 °C. After 1 h when the OD_{600} had reached 1.5, 0.1 mM IPTG was added to induce Neu5Ac adolase production for 5 h. Cells were harvested by centrifugation at 5,000 × g for 10 min, and washed twice by 0.85% NaCl solution. The activity of NanA was assayed according to the previous work.[14] One unit of NanA activity was defined as the amount of NanA which catalyzed the production of 1 μmol pyruvate per min at 37 °C.

Preparation of *Pseudomonas stutzeri* SDM Cells

The bacterial strain *Pseudomonas stutzeri* SDM, capable of oxidizing lactate to pyruvate, was isolated from soil by our laboratory and deposited at the China Center for Type Culture Collection (CCTCC No. M 206010). Cells of strain SDM (18.1 g wet weight, about 907 U of LOX) were cultured and harvested from 1,000 mL medium according to the previous study.[8a] One unit (U) of the LOX from lactate was defined as the amount of LOX required for the formation of 1.0 μmol of pyruvate per minute under the test condition.

Preparation of the ManNAc/GlcNAc Mixture

The procedure of base-catalyzed epimerization of GlcNAc to ManNAc was adopted to prepare ManNAc.[3b,e,15] Typically, 400 g of GlcNAc was dissolved in 1 L of NaOH (55 mM) aqueous solution and allowed to stand at 25 °C for 48 h. The ManNAc:GlcNAc ratio was approximately 3.5:1.[15a] The resulting pale yellow solution was neutralized by the addition of glacial acetic acid and then air-dried under vacuum. The residue was reflux extracted by 500 mL methanol for 1 h. The extracted solution was allowed at 25 °C for 4 h, and filtered to remove GlcNAc. The filtrate was concentrated under reduced pressure to provide a mixture of GlcNAc/ManNAc. The final mixture of ManNAc/GlcNAc[3e,15a] was analyzed, and the ratio of ManNAc:GlcNAc was about 5.67:1. In each batch, 293.43 ± 2.66 g GlcNAc was recovered. The recovered GlcNAc could be reused in the next cycle. About 95.37 ± 10.52 g mixture containing 85.01 ± 3.13% of ManNAc was obtained from 400 g GlcNAc.

Synthesis of Neu5Ac

The production of Neu5Ac was carried out in one pot. The reaction mixture in a 3,000-mL flask contained cells of *E. coli* BL21 (DE3) (pET15b-*nan*A) (2.1 g in wet weight, about 625 U NanA), cells of strain SDM (14.5 g in wet weight, about 727 U of LOX), 19.62 g ManNAc/GlcNAc mixture, 26.25 g L-lactate (adjusted to pH 7.0 with NaOH), 4 mL toluene (to permeabilize the cells), and 16 mL 1 M Tris-HCl (pH = 7.0). Then, deionized water was added to bring the total volume to 400 mL. Reference batches with only one type of cells contained the same amount of substrates and other materials dispersed in water. One is Control A which contained only strain SDM, and the other is Control B which contained only strain *E. coli* BL21 (DE3) (pET15b-*nan*A). The reaction was carried out at 30 °C with agitation (200 rpm) for 16–40 h. Triplicate experiments were performed under the same conditions.

Isolation of Neu5Ac after Biotransformation

After biotransformation for 20 h, the reaction solution was centrifuged at 5,000 × g for 10 min to remove pellets, and the supernatant was adjusted to pH 4.5 with glacial acetic acid. Then, the supernatant was loaded onto an anionic resin column (800 mL) which had been pre-treated with 2 M formic acid. The unreacted GlcNAc and ManNAc were washed clear with deionized water. Neu5Ac was eluted with a linear gradient of 0–1 M formic acid in 160 min with the flow rate at 10 mL min^{-1}. The desired fraction was concentrated to approximately 125 g/liter Neu5Ac on a rotary evaporator. Neu5Ac was crystallized by adding 5 volumes of glacial acetic acid. After standing for 2 days at 4 °C, the solid part was recovered by vacuum filtration and washed with fresh glacial acetic acid before drying at 40 °C to a constant weight.

Acknowledgements

The work was supported in part by the grant from the State Major Basic Research Development Program (China) (Grant 2007CB707800) and the State Key Foundation of the Ministry of Education of China (Grant no. 106102). We would like to acknowledge the financial support and chemical analysis by Shanghai Apple Flavor & Fragrance Co., Ltd. and Shanghai Jiao Tong University (China).

References

[1] a) R. Schauer, *Arch. Biochem. Biophys.* **2004**, *426*, 132–141.

[2] a) M. Koketsu, H. Kawanami, L. J. Juneja, M. Jujiki, H. Hatta, K. Nishimoto, M. Kim, N. Yamazaki, (Kaiyo Kagaku Co., Ltd.), *U.S. Patent* 5,233,033, **1993**; b) E. J. McGuire, S. B. Binkley, *Biochemistry* **1964**, *170*, 247–251; c) M. Shimatani, Y. Uchida, I. Matsuno, M. Oyoshi, Y. Ishiyama, (Snow Brand Milk Products Co. Ltd.), *U.S. Patent* 5,270,462, **1993**.

[3] a) C. Auge, S. David, C. Gautheron, *Tetrahedron Lett.* **1984**, *25*, 4663–4664; b) S. Blayer, J. M. Woodley, M. J. Dawson, M. D. Lilly, *Biotechnol. Bioeng.* **1999**, *66*, 131–136; c) M. J. Kim, W. A. J. Hennen, H. M. Sweers, C. H. Wong, *J. Am. Chem. Soc.* **1988**, *110*, 6481–6484; d) U. Kragl, D. Gygax, O. Ghisalba, C. Wandrey, *Angew. Chem. Int. Ed.* **1991**, *30*, 827–828; e) M. Mahmoudian, D. Noble, C. S. Drake, R. F. Middleton, D. S. Montgom-

ery, J. E. Piercey, D. Ramlakhan, M. Todd, M. J. Dawson, *Enzyme Microb. Technol.* **1997**, *20*, 393–400.
[4] M. P. DeNinno, *Synthesis* **1991**, 583–593.
[5] a) K. Aisaka, T. Uwajima, *Appl. Environ. Microbiol.* **1986**, *51*, 562–565; b) C. Karen, D. Kenneth, E. G. Gary, *Mol. Biochem. Parasitol.* **1996**, *76*, 289–292; c) G. G. Lilley, M. Von Itzstein, N. Ivancic, *Protein Expression Purif.* **1992**, *3*, 434–440; d) Y. , M. Ohta, Shimosaka, K. Murata, M. Shimosaka, K. Murata, Y. Tsukada, A. Kimura, *Appl. Microbial. Biotechnol.* **1986**, *24*, 386–391.
[6] H. E. Schomaker, D. Mink, M. G. Wubbolts, *Science* **2003**, *299*, 1694–1697.
[7] C. Q. Ma, P. Xu, Y. M. Dou, Y. B. Qu, *Biotechnol. Prog.* **2003**, *19*, 1672–1676.
[8] a) C. Q. Ma, P. Xu, J. H. Qiu, Z. J. Zhang, K. W. Wang, M. Wang, Y. N. Zhang, *Appl. Microbiol. Biotechnol.* **2004**, *66*, 34–39; b) P. Xu, T. Yano, K. Yamamoto, H. Suzuki, H. Kumagai, *J. Ferment. Bioeng.* **1996**, *81*, 357–359; c) P. Xu, T. Yano, K. Yamamoto, H. Suzuki, H. Kumagai, *Appl. Biochem. Biotechnol.* **1996**, *56*, 277–288; d) J. R. Hao, C. Q. Ma, C. Gao, J. H. Qiu, M. Wang, Y. N. Zhang, X. Cui, P. Xu, *Biotechnol. Lett.* **2007**, *29*, 105–110.
[9] G. G. Lilley, J. R. G. Barbosa, L. A. Pearce, *Protein Expression Purif.* **1998**, *12*, 295–304.
[10] S. Koizumi, K. Tabata, T. Endo, A. Ozaki, (Kyowa Hakko Kogyo Co., Ltd.), *U.S. Patent* 6,846,656, **2005**.
[11] J. H. Andrew, *Bioorg. Med. Chem.* **2002**, *10*, 3175–3185.
[12] H. Yu, H. Yu, R. Karpel, X. Chen, *Bioorg. Med. Chem.* **2004**, *12*, 6427–6435.
[13] Y. Ohta, K. Watanabe, A. Kimura, *Nucleic Acids Res.* **1985**, *13*, 8843–8852.
[14] P. Brunetti, G. W. Jourdian, S. Roseman, *J. Biol. Chem.* **1962**, *237*, 2447–2453.
[15] a) J. Song, H. S. Zhang, B. Y. Wu, Y. X. Zhang, H. F. Li, M. Xiao, P. G. Wang, *Mar. Drugs.* **2003**, *1*, 34–45; b) C. T. Spivak, S. Roseman, *J. Am. Chem. Soc.***1959**, *81*, 2403–2404.

