Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 847 (2007) 82–87
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Assessment of 4-nitro-1,8-naphthalic anhydride reductase activity in homogenates of bakers' yeast by reversed-phase high-performance liquid chromatography

Jianqiang Xu [a,b,1], Qing Yang [a,2], Xuhong Qian [c], Jörgen Samuelsson [b], Jan-Christer Janson [b,*]

[a] *Department of Bioscience and Biotechnology, Dalian University of Technology, Dalian 116024, China*
[b] *Department of Surface Biotechnology, Uppsala Biomedical Center, Uppsala University, P.O. Box 577, Uppsala SE-751 23, Sweden*
[c] *Shanghai Key Laboratory of Chemical Biology, School of Pharmacy, East China University of Science and Technology, Shanghai 200237, China*

Received 4 April 2006; accepted 15 September 2006
Available online 27 October 2006

Abstract

A simple reversed-phase high-performance liquid chromatographic (RP-HPLC) method was developed for the simultaneous determination of yield and conversion ratio of 4-nitro-1,8-naphthalic anhydride to 4-amino-1,8-naphthalic anhydride following incubation with a crude bakers' yeast homogenate. The analytes were separated on a C18 column in gradient mode. The detection limit of 4-amino-1,8-naphthalic anhydride is 10 ng/μl when using a 10 μl sample injection volume. The nitroreductase activity in the homogenate system can be assessed during the bioconversion process. The method can be used for the simultaneous detection of 4-hydroxylamino-1,8-naphthalic anhydride, an intermediate with limited stability.

Keywords: Nitroaryl; Nitroarene; Arylhydroxylamine; Aminoaryl; Bakers' yeast; Nitroarylreductase; HPLC; TLC

1. Introduction

Microorganisms possessing the ability to reduce a wide range of nitroarene compounds are known [1,2] and have become valuable assets in biodegradation of nitro explosives [3–9], bioremediation of other nitroaromatic pollutants [10,11], and in biotransformation processes [12–18]. Nitroarenes, arylhydroxylamines and arylamines are important intermediates in the industrial manufacturing of dyes, pesticides and plastics, and are significant environmental pollutants. In recent years, increasing concern of ecological and health threats posed by nitroaromatics has attracted much attention and encouraged the development of sensitive methods for their detection followed by microorganism-mediated biodegradation/bioremediation [19–25]. Recent investigations have demonstrated the usefulness of bakers' yeast for the reduction of nitroarene compounds to their corresponding amines in chemoselective bioorganic synthesis [26–29]. It is well known that in non-enzymatic chemical nitroreduction processes, arylhydroxylamines are unstable and easily convert to amine [30].

Enzymatic reduction of aromatic nitro compounds to the corresponding arylhydroxylamines by bakers' yeast has recently been reported [31]. However, there are examples in the literature that nitroarylreductases (NTR) from other sources also possess this ability [32–40]. In addition, several enzymes including cytosolic nitroreductase [41–43], xanthine oxidase [44–46], aldehyde oxidase [47,48], cytochrome NAD(P)H:P450 reductase [49,50], and DT-diaphorase (NAD(P)H:quinone oxidoreductases) [51–53] were proposed to participate in the reduction

Abbreviations: NTR, nitroarylreductase; 4-ANA, 4-amino-1,8-naphthalic anhydride; 4-HANA, 4-hydroxylamino-1,8-naphthalic anhydride; 4-NNA, 4-nitro-1,8-naphthalic anhydride; RP-HPLC, reversed-phase high-performance liquid chromatography; TLC, thin-layer chromatography

* Corresponding author. Tel.: +46 70 5565088; fax: +46 18 555016.
E-mail addresses: qingyang@dlut.edu.cn (Q. Yang), jan-christer.janson@ytbioteknik.uu.se (J.-C. Janson).

[1] Experimental work was performed in the Department of Surface Biotechnology, Uppsala Biomedical Center, Uppsala University, Sweden.

[2] Present address: Department of Cell Biology, Harvard Medical School, Harvard University, Boston, MA 02115, United States.

1570-0232/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2006.09.040

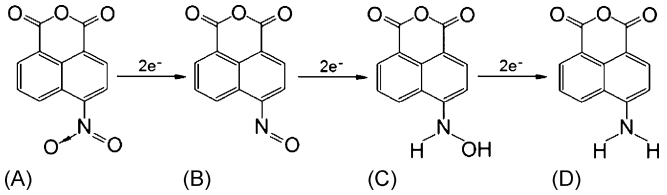

Fig. 1. Proposed pathway of the 4-NNA nitroreduction reaction by crude homogenates from bakers' yeast. (A) 4-nitro-1,8-naphthalic anhydride; (B) 4-nitroso-1,8-naphthalic anhydride; (C) 4-hydroxylamine-1,8-naphthalic anhydride; (D) 4-amine-1,8-naphthalic anhydride.

of a variety of nitroaromatic substrates to the corresponding hydroxylamines. Based on the known metabolism of nitroarene compounds by nitroreductase [2,4,39], a plausible description of the reaction is as follows: The nitroarene compound is first reduced via two electrons to form an intermediate which accepts another two electrons to form an arenehydroxylamine. Finally the arenehydroxylamine is converted to amine by a two electron reaction (see Fig. 1). For instance, in the 3-nitrobenzanthrone (3-NBA) activation process, Arlt et al. [54] proposed that several cytosolic reductases participate in the initial nitroreduction to produce an unstable 3-hydroxylaminobenzanthrone (3-HABA). Further reduction of the hydroxylamine will result in the formation of a stable amine.

Successful purification and characterization of nitroreductases from bakers' yeast, followed by cloning and expression in a high-producing host, such as *Pichia pastoris*, could facilitate the establishment of a technical process for the bioconversion of valuable intermediates to useful final products, for the environment-friendly elimination of nitroaromatics (e.g. TNT), and perhaps even to gene-directed enzyme prodrug therapy (GDEPT) [55–57].

The simplicity, efficiency and reproducibility of liquid chromatography, has led to numerous applications for the determination and quantification of compounds and/or their metabolites using UV–vis [58–61] or fluorescence detection [62–64] and for the assessment of enzymatic activity [64–74]. Recently, Chen et al. [64] established methods for the detection of 3-NBA reductase activity in mammalian tissues using normal-phase HPLC coupled to the measurement of a fluorogenic amine. However, no enzymatic assay has yet been reported for the measurement of bioreduction of aromatic nitro compounds to arylhydroxylamines. Neither any report describing a method to study the bioreduction process with the intermediate formation of hydroxylamine.

Initially, attempts were made to develop a spectrophotometric assay for nitroreductase activity in bakers' yeast homogenates using 4-nitro-1,8-naphthalic anhydride (4-NNA) as substrate, but no 4-hydroxylamino-1,8-naphthalic anhydride could be detected. This paper describes a simple specific method for assessing 4-NNA reductase activity applicable to homogenates of bakers' yeast or any other source. The procedure involves reversed-phase high-performance liquid chromatography (RP-HPLC) and thin-layer chromatography (TLC), which have proved to be inexpensive, sufficiently sensitive and reproducible methods for the detection and quantification of 4-ANA.

2. Experimental

2.1. Chemicals and equipment

4-NNA, 95% purity (CAS34087-02-0), was purchased from Sigma–Aldrich. Custom synthesized 4-ANA was a gift by Dr. Chuanzheng Zhou (Department of Bioorganic Chemistry, Uppsala BMC, Uppsala University, Uppsala, Sweden). Biosynthesized 4-HANA was a generous gift by Prof. Xuhong Qian (East China University of Science and Technology, Shanghai, China). The solvents methanol, ethanol, ethyl acetate, and *n*-hexane were all HPLC-grade and purchased from Merck AG, Darmstadt, Germany. Dimethylsulfoxide (DMSO), reduced nicotinamide adenine dinucleotide (β-NADH), nicotinamide adenine dinucleotide phosphate (β-NADPH), FAD and FMN were obtained from Sigma–Aldrich. Milli-Q water was used in all experiments. All other chemicals and solvents were of analytical or HPLC grade. All reversed-phase HPLC experiments were performed at ambient temperature (26 °C ± 1 °C) using a Kromasil C18 column (4.6 mm × 250 mm i.d.; 5 μm, 80 Å) attached to an ÄKTA Basic workstation (GE Healthcare Bio-Sciences, Uppsala, Sweden), equipped with pump P-900, monitor UV-900, fraction collector Frac-900, and Unicorn 4.10 software. UV-900 is a three-wavelength UV–vis monitor that uses advanced fiber optic technology to monitor with high sensitivity at up to three wavelengths simultaneously in the wavelength range 190–700 nm.

2.2. Synthesis of 4-ANA

4-ANA was synthesized after modification of a published procedure [75]. In brief, 4-NNA (24.3 mg, 0.1 mmol) and iron powder (33.6 mg, 0.6 mmol) were added to 10 ml acetic acid and the reaction mixture was stirred at reflux temperature for 20 h. After cooling to room temperature the reaction mixture was extracted five times with 25 ml ethyl acetate. After filtration the extract was evaporated under reduced pressure and the crude reaction product could be recovered as an orange colored solid (8.68 mg, 0.04 mmol, 40% yield). The crude product was purified by silica gel chromatography in *n*-hexane:ethyl acetate (10:1, v/v). The structure and the molecular weight of the pure 4-ANA were verified by ^{1}H NMR, ^{13}C NMR and LC–MS, respectively, and its purity was checked by RP-HPLC.

2.3. Synthesis of 4-HANA

Plant cells have been used in biotransformation studies [76,77] and recently as biocatalysts for aromatic nitroreduction [78]. In this work, 4-HANA was synthesized according to a published procedure [78]. Briefly, fresh grape tissue (*Vitis vinifera* L.) were washed thoroughly with 75% ethanol (v/v) and then with distilled water in order to remove possible microbial contamination. After the washing steps, the grape tissue was immediately homogenized using a sterilized knife. Finally, 100 mg 4-NNA was added to a suspension of 60 g homogenate in 100 ml of water, and the mixture was stirred at 25 °C for 4 days. The bioconversion reaction was terminated by the addition of 100 ml

ethyl acetate. After a three-fold extraction procedure the separated organic phases were combined and filtered through a celite pad followed by drying over anhydrous $MgSO_4$. After removal of the organic solvent by vacuum evaporation, the residue was dissolved again in a proper volume of ethyl acetate and was purified by silica flash chromatography in *n*-hexane:ethyl acetate (5:1 v/v) to give pure hydroxylamine with a conversion yield of 75%. The structure and the molecular weight of the 4-HANA were verified by ^{1}H NMR, ^{13}C NMR and LC–MS, respectively.

2.4. Preparation of yeast homogenates

Fifty gram (wet cell weight) of bakers' yeast was suspended in 100 ml 20 mM Tris–HCl (pH 7.0) and disrupted by repeated passages though a French pressure cell. The suspension was centrifuged at 13,000 × *g* for 1 h at 4 °C and the clarified homogenates stored at −20 °C for further use. The protein concentration was estimated using the Bradford method [79] with bovine serum albumin (BSA) as reference standard.

2.5. Nitroreduction reaction

The nitroreduction reaction took place in 150 ml E-flasks shaken 100 rpm at 26 °C. The reaction mixture was composed of 4.5 ml 0.2 M Na phosphate buffer, pH 6.5, containing 0.2 ml 4-NNA (25 mg/ml), 0.3 ml NADPH (10 mM) and 5 ml fresh crude bakers' yeast homogenate containing 100 mg protein. After varying periods of time the reaction of one of the flasks was terminated by the addition of 100 ml ethyl acetate. After a three-fold extraction procedure the separated organic phases were combined and filtered through a celite pad followed by drying over anhydrous $MgSO_4$. After removal of the organic solvent by vacuum evaporation, the residue was dissolved in 2 ml ethyl acetate and the amount of 4-ANA formed determined by reversed-phase HPLC. All calibration standards and samples were dissolved in ethyl acetate.

2.6. UV–vis spectrophotometric analysis

In Fig. 2 are shown the UV–vis absorbance spectra of 4-NNA, 4-ANA and 4-HANA, respectively. The specific absorbance wavelengths of 4-NNA (in methanol) are 230 and 341 nm in the ultraviolet range (Fig. 2 black line). The specific absorbance wavelengths of 4-ANA were 223 and 271 nm in the ultraviolet range and 424 nm in the visible range (Fig. 2 red line). Similarly, the specific absorbance wavelengths of 4-HANA were 226 and 268 nm in the ultraviolet range and 424 nm in the visible range (Fig. 2 blue line). In this study, 4-NNA (with strong UV-absorption) is enzymatically converted to 4-HANA and then to 4-ANA both of which possess weak UV absorption at wavelengths in the range 280–400 nm (Fig. 2). In parallel a significant decrease in the signals at 254 and 346 nm was observed. The significant changes in UV and visible spectra indicate that the 4-NNA had been biotransformed to 4-ANA under aerobic conditions (Fig. 2). The synthesized product (4-ANA) had a purity >98% as analyzed by RP-HPLC (data not shown).

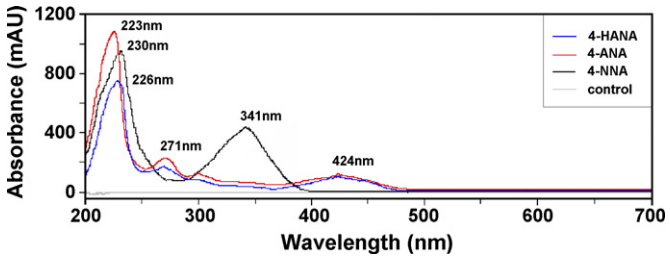

Fig. 2. UV–vis spectra of 4-NNA, 4-ANA and 4-HANA in methanol. For 4-ANA: peak 1, 223 nm; peak 2, 271 nm; peak 3, 424 nm; for 4-HANA: peak 1, 226 nm; peak 2, 268 nm; peak 3, 424 nm; for 4-NNA: peak 1, 230 nm; peak 2, 341 nm.

2.7. Reversed-phase HPLC detection

The C18 column was equilibrated with 0.1% (w/v) citric acid in deionized water (solvent A) containing 30% solvent B (0.1% (w/v) citric acid in methanol) for 25 min at a flow-rate of 0.8 ml/min. After sample injection (10 μl) elution was achieved in gradient mode to 100% solvent B in 60 min, maintaining 100% solvent B for 15 min, and returning to 30% solvent B for 25 min. The integrating multi-wavelength detector was set at 424 nm for detection of 4-ANA and 4-HANA, at 346 nm for detection of residual 4-NNA and at 254 nm for detection for all components in the reaction mixture.

2.8. Calibration curve of 4-ANA and its detection limit

A 4-ANA calibration curve in ethyl acetate was prepared using the following sample concentrations (ng/μl): 6, 12, 24, 48, 96, 182, 364, 728, 1378, 2050 and 2700. Calibration standards in ethyl acetate were prepared immediately before each calibration run. Ten microliters of each sample was injected into the Kromasil C18 column connected to the ÄKTA basic HPLC system and monitored at 424 nm. The amount of 4-ANA was calculated from the integrated area of the peak with a retention time of 20 min. The calibration curve equation is $Y = AX$, where Y represents the integral area of the 4-ANA peak and X the concentration of 4-ANA in ng/μl. A linear calibration curve was obtained for concentrations between 6 and 2.7 μg/μl ($Y = 38.729X$, $R^2 = 0.9981$). The detection limit of 4-ANA is 10 ng/μl when using a 10 μl sample injection volume.

2.9. Thin-layer chromatography analysis

The solvent mixture *n*-hexane:ethyl acetate (50:50, v/v) was used for normal-phase thin-layer chromatography. Samples were analyzed for residual 4-NNA and its biotransformation products using visual fluorescence detection. The visible, UV and fluorescent pictures of the products in TLC flat were captured by using Kodak DZ7440 digital camera with color science chip, under the irradiation of UV light at 254 and 346 nm, respectively.

3. Results and discussion

3.1. Detection of reaction products

The use of fluorogenic substrates for the detection of microbial nitroreductases has been published [80]. In the present work, commercially available 4-NNA (a non-fluorescent light yellow powder having strong UV-absorption) was used as substrate for the biotransformation and for the chemical synthesis of the fluorescent 4-ANA and 4-HANA. In the enzymatic process, 4-NNA is converted to the intermediate 4-HANA and then to 4-ANA, both of which possess strong yellow fluorescence caused by the conjugated structure composed of the naphthalic anhydride ring and the amino group. The formation of fluorescent products, visible in normal-phase thin-layer chromatography, supports the results from the RP-HPLC analysis. However, TLC analysis cannot distinguish between the unstable 4-HANA intermediate and the final product 4-ANA. This is why the separation and isolation of 4-HANA using flash chromatography on neat silica is difficult to conduct. Trace impurities were detected in both 4-NNA (<2%) and in the biotransformed product as analyzed by thin-layer chromatography and visual fluorescence detection (data not shown).

3.2. Reversed-phase HPLC analysis of 4-NNA, 4-HANA and 4-ANA

After the ethyl acetate extraction, the biotransformation products were separated on a Kromasil C18 column in gradient mode as described above (Section 2.8). The retention times of 4-HANA, 4-ANA and 4-NNA were 19.5, 20.5 and 26.7 min, respectively (Fig. 3). No UV adsorption was observed for 4-NNA at 424 nm.

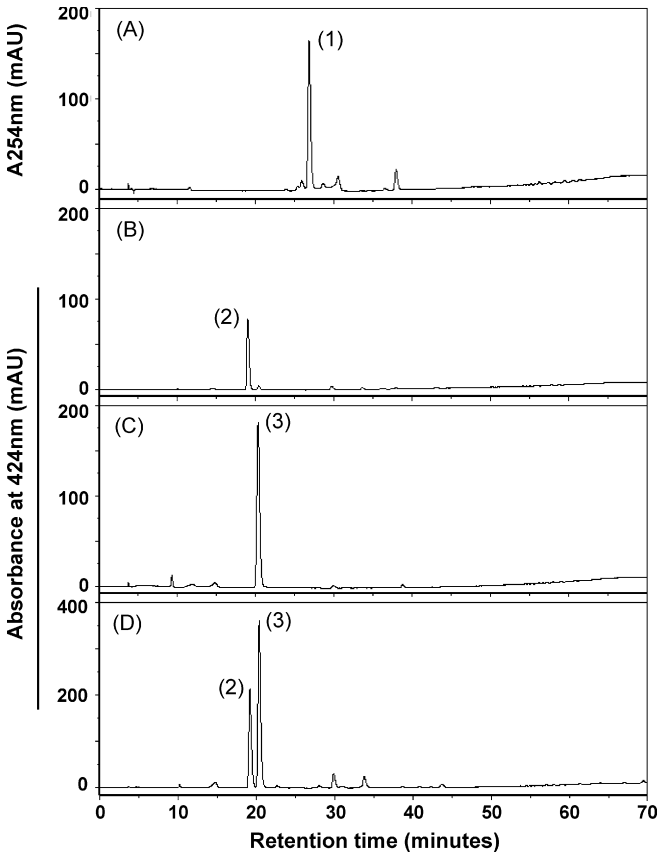

Fig. 3. RP-HPLC analysis of 4-NNA, 4-ANA and 4-HANA using a Kromasil C18, 5 μm column. (A) Reference sample of 4-NNA, retention time 26.7 min; (B) Reference sample of 4-HANA, retention time 19.5 min; (C) Reference sample of 4-ANA, retention time 20.5 min; (D) Crude product mixture after incubation with whole-cell bakers' yeast; peaks: (1) 4-NNA; (2) 4-HANA; (3) 4-ANA.

3.3. Time-course of biotransformation using a crude bakers' yeast homogenate

In Fig. 4 (chromatograms A–F) the biotransformation of 4-NNA to 4-ANA using a crude bakers' yeast homogenate is followed by RP-HPLC analysis at 24 h intervals. In the chromatogram, recorded at 424 nm, a 4-ANA peak with a retention time of 20 min appeared after 48 h incubation (Fig. 4B). Maximum production of 4-ANA was reached after incubation of 4-NNA for 96 h (Fig. 4D). The total 144 h long time-course detection of nitroreduction reaction exhibited a dynamic process from 4-NNA to 4-ANA with a trace of the unstable intermediate 4-HANA recorded at 48 h. The scatter in the amount of 4-ANA produced (it is expected to reach a plateau rather than a maximum) can be explained by a variation in the incubation conditions between the individual reaction flasks.

3.4. 4-HANA stability

It is known that the reduction of nitro compounds to amine proceeds through an intermediate stage involving hydroxylamine derivatives. These are extremely unstable under chemical reduction conditions and once regarded to be non-detectable. Our additional interest in arylhydroxylamine generation encouraged us to develop a simple method to follow the dynamic process from arylhydroxylamine to amine. In this study, we found a way to detect concomitantly generated hydroxylamine intermediate besides amine and to follow the dynamic generation of amine from unstable hydroxylamine in aqueous phase (50% methanol, v/v) using RP-HPLC (Fig. 5). The possible intermediate 4-nitroso-1,8-naphthalic anhydride could not be detected

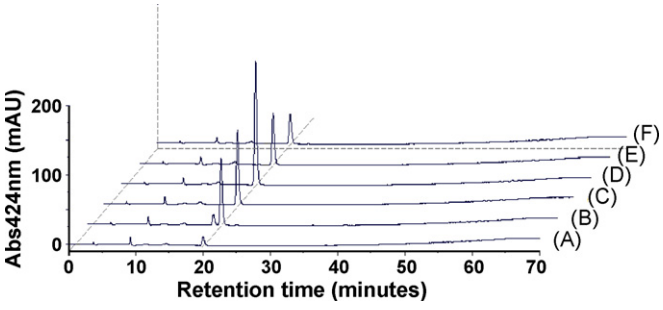

Fig. 4. Time course RP-HPLC analysis of the formation of 4-ANA and 4-HANA from 4-NNA during incubation with crude bakers' yeast homogenate. Incubation time: (A) 24 h, (B) 48 h, (C) 72 h, (D) 96 h, (E) 120 h; (F) 144 h. Detection wavelength 424 nm.

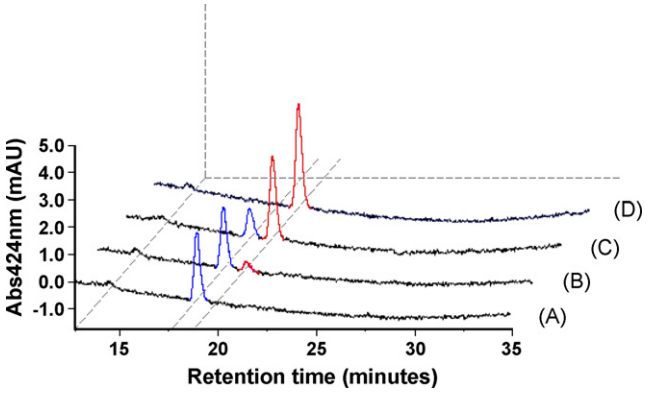

Fig. 5. RP-HPLC composition analysis of the reaction mixture during the formation of stable amine (red peak) from unstable hydroxylamine (blue peak) in the presence of 50% (v/v) methanol at room temperature. (A) Original 4-HANA sample; (B) After 4 days storage; (C) After 8 days storage; (D) Final 4-ANA sample.

and 4-HANA was gradually further reduced to the amine, suggesting they were unstable intermediates in the reaction.

4. Conclusions

A simple method combining reversed-phase HPLC and thin-layer chromatography has been described to assess 4-nitro-1,8-naphthalic anhydride nitroreductase activity in bakers' yeast homogenates by quantifying the formation rate of the final product 4-amino-1,8-naphthalic anhydride in an aqueous reaction system. The method has proved its applicability in the analysis of 4-amino-1,8-naphthalic anhydride and is thus suitable for monitoring nitroreduction and amine nitroreductase activity. Although the identification of the enzymes involved in the nitroarene reduction process in bakers' yeast has not yet been established, the method reported here could contribute to further investigations of the nitroreduction mechanism.

Acknowledgements

Financial support by the Swedish government through its agency VINNOVA is gratefully acknowledged. Partial assistant under the auspices of Grant 20576016 from National Natural Science Foundation of China and Grant 101072 from Fok Ying Tong Education Foundation and Ministry of Education of China. The help by Dr. Chuanzheng Zhou (Department of Bioorganic Chemistry, Uppsala BMC, Uppsala University, Sweden) for the 4-ANA standard synthesis, TLC detection and product identification, is greatly appreciated. We also thank Professor Jin-Ping Li (Department of Microbiology and Medical Biochemistry, Uppsala BMC, Sweden) and Professor Jin-Nan Cui (Department of Fine Chemistry, Dalian University of Technology, Dalian, China) for useful discussions.

References

[1] R.E. Asnis, Arch. Biochem. Biophys. 66 (1957) 208.
[2] J.C. Spain, Ann. Rev. Microbiol. 49 (1995) 523.
[3] A. Esteve-Núñez, A. Caballero, J.L. Ramos, Microbiol. Mol. Biol. Rev. 65 (2001) 335.
[4] H. Yin, T.K. Wood, B.F. Smets, Appl. Microbiol. Biotechnol. 67 (2005) 397.
[5] J.L. Romos, M.M. González-Pérez, A. Caballero, P.van. Dillewijn, Curr. Opin. Biotechnol. 16 (2005) 1.
[6] C.A.F. Aiub, J.L. Mazzei, L.F.R. Pinto, I. Felzenszwalb, Chem. Biol. Interact. 161 (2006) 146.
[7] T.J. Fleischmann, K.C. Walker, J.C. Spain, J.B. Hughes, A.M. Craig, Biochem. Biophys. Res. Commun. 314 (2004) 957.
[8] T.A. Lewis, D.A. Newcombe, R.L. Crawford, J. Environ. Manage. 70 (2004) 291.
[9] S.F. Nishino, G.C. Paoli, J.C. Spain, Appl. Environ. Microbiol. 66 (2000) 2139.
[10] S.A. Zaripov, A.V. Naumov, J.F. Abdrakhmanova, A.V. Garusov, R.P. Naumova, FEMS Microbiol. Lett. 217 (2002) 213.
[11] H.Y. Kim, G.N. Bennett, H.G. Song, Biotechnol. Lett. 24 (2002) 2023.
[12] J. Hawari, A. Halasz, S. Beaudet, L. Paquet, G. Ampleman, S. Thiboutot, Appl. Environ. Microbiol. 65 (1999) 2977.
[13] C.L. Davey, L.W. Powell, N.J. Turner, A. Wells, Tetrahedron Lett. 35 (1994) 7867.
[14] R.P. Naumova, T.O. Belousova, R.M. Giliazova, Prikl. Biokhim. Mikrobiol. 18 (1982) 85.
[15] H.L. Holland, Curr. Opin. Chem. Biol. 2 (1998) 77.
[16] P.D. Fiorella, J.C. Spain, Appl. Environ. Microbiol. 63 (1997) 2007.
[17] T.A. Khan, R. Bhadra, J. Hughes, J. Ind. Microbiol. Biotechnol. 18 (1997) 198.
[18] C.A. Metosh-Dickey, R.P. Mason, G.W. Winston, Toxicol. Appl. Pharmacol. 154 (1999) 126.
[19] W.D. Won, L.H. DiSalvo, J. Ng, Appl. Environ. Microbiol. 31 (1976) 576.
[20] R.J. Spanggord, K.E. Mortelmans, A.F. Griffin, V.F. Simmon, Environ. Mutagen. 4 (1982) 163.
[21] E.L. Tan, C.H. Ho, W.H. Griest, R.L. Tyndall, J. Toxicol. Environ. Health 36 (1992) 165.
[22] M.E. Honeycutt, A.S. Jarvis, V.A. McFarland, Ecotoxicol. Environ. Saf. 35 (1996) 282.
[23] J.S. Fucsh, M.L. Oneto, N.B. Casabe, S.O. Comez, R. Tarulla, M. Vaccarezza, C. Sanchez-Rivas, E.M. Kesten, E.J. Wood, Bull. Environ. Contam. Toxicol. 67 (2001) 696.
[24] S. Homma-Takeda, Y. Hiraku, Y. Ohkuma, S. Oikawa, M. Murata, K. Ogawa, T. Iwamuro, S. Li, G.F. Sun, Y. Kumagai, N. Shimojo, S. Kawanishi, Free Radic. Res. 36 (2002) 555.
[25] J.A. Steevens, B.M. Duke, G.R. Lotufo, T.S. Bridges, Environ. Toxicol. Chem. 21 (2002) 1475.
[26] W. Baik, J.L. Han, K.C. Lee, B.H. Kim, J.T. Hahn, Tetrahedron Lett. 35 (1994) 3965.
[27] W. Baik, J.U. Rhee, S.H. Lee, N.H. Lee, B.H. Kim, K.S. Kim, Tetrahedron Lett. 36 (1995) 2793.
[28] W. Baik, T.H. Park, J. Org. Chem. 60 (1995) 5683.
[29] J.A. Blackie, N.J. Turner, A.S. Wells, Tetrahedron Lett. 38 (17) (1997) 3043.
[30] J.L. Bolton, R.A. McClelland, J. Am. Chem. Soc. 111 (1989) 8172.
[31] F. Li, J.N. Cui, X.H. Qian, R. Zhang, Chem. Commun. 20 (2004) 2338.
[32] C.S. Tsai, Biochem. J. 242 (1987) 447.
[33] T. Kinouchi, Y. Ohnishi, Appl. Environ. Microbiol. 46 (1983) 596.
[34] C. Bryant, M. DeLuce, J. Bacteriol. 266 (1991) 4119.
[35] H.Y. Kim, H.G. Song, Appl. Microbiol. Biotechnol. 68 (2005) 766.
[36] S. Rieble, D.K. Joshi, M.H. Gold, Biochem. Biophys. Res. Commun. 205 (1994) 298.
[37] F. Rafii, G. Hehman, P. Lunsford, Arch. Microbiol. 176 (2001) 381.
[38] F.J. Peterson, R.P. Mason, J. Hovsepian, J.L. Holtzman, J. Biol. Chem. 254 (1979) 4009.
[39] R.L. Koder, C.A. Haynes, M.E. Rodgers, D.W. Rodgers, A.F. Miller, Biochemistry 41 (2002) 14197.
[40] J. Rau, A. Stolz, Appl. Environ. Microbiol. 69 (2003) 3448.
[41] L.R. Kober, A.F. Miller, Biochim. Biophys. Acta 1387 (1998) 395.
[42] S. Zenno, H. Koike, M. Tanokura, K. Saigo, J. Biochem. (Tokyo) 120 (1996) 736.

[43] G.N. Parkinson, J.V. Skelly, S. Neidle, J. Med. Chem. 43 (2000) 3624.
[44] E.D. Clarke, P. Wardman, K.H. Goulding, Biochem. Pharmacol. 29 (1980) 2684.
[45] S.L. Bauer, P.C. Howard, Cancer Lett. 54 (1990) 37.
[46] P. Reigan, P.N. Edwards, A. Gbaj, C. Cole, S.T. Barry, K.M. Page, S.E. Ashton, R.W.A. Luke, K.T. Douglas, I.J. Stratford, M. Jaffar, R.A. Bryce, S. Freeman, J. Med. Chem. 48 (2005) 392.
[47] B.F. Dudley, G.W. Winston, Arch. Biochem. Biophys. 316 (1995) 879.
[48] M. Stiborová, M. MiksŤanová, S. Smrček, C.A. Bieler, A. Breuer, K.A. Klokow, H.H. Schmeiser, E. Frei, Carcinogenesis 25 (2004) 833.
[49] V.M. Arlt, H. Glatt, E. Muckel, U. Pabel, B.L. Sorg, A. Seidel, H. Frank, H.H. Schmeiser, D.H. Phillips, Int. J. Cancer 105 (2003) 583.
[50] V.M. Arlt, A. Hewer, B.L. Sorg, H.H. Schmeiser, D.H. Phillips, M. Stiborova, Chem. Res. Toxicol. 17 (2004) 1092.
[51] K.B. Wu, R.J. Knox, X.Z. Sun, P. Joseph, A.K. Jaiswal, D. Zhang, P.S.K. Deng, S. Chen, Arch. Biochem. Biophys. 347 (1997) 221.
[52] J. Šarlauskas, E. Dičkancaiteg, A. Nemeikaiteg, Ž. Anusevičius, H. Nivinskas, J. Segura-Aguilar, N. Čegnas, Arch. Biochem. Biophys. 346 (1997) 219.
[53] J.V. Skelly, R.J. Knox, T.C. Jenkins, Mini Rev. Med. Chem. 1 (2001) 293.
[54] V.M. Arlt, H. Glatt, E. Muckel, U. Pabel, B.L. Sorg, H.H. Schmeiser, D.H. Phillips, Carcinogenesis 11 (2002) 1937.
[55] Z.R. Li, J.Y. Han, Y.Y. Jiang, P. Browne, R.J. Knox, L.Q. Hu, Bioorg. Med. Chem. 11 (2003) 4171.
[56] M.P. Hay, W.R. Wilson, W.A. Denny, Bioorg. Med. Chem. 13 (2005) 4043.
[57] Y.Y. Jiang, J.Y. Han, C.Z. Yu, S.O. Vass, P.F. Searle, P. Browne, R.J. Knox, L.Q. Hu, J. Med. Chem. 49 (2006) 4333.
[58] S.D. Francia, E. Pirro, F. Zappia, F.D. Martino, A.E. Sprio, F. Daffara, M. Terzolo, A. Berruti, F.D. Carlo, F. Ghezzo, J. Chromatogr. B 837 (2006) 69.
[59] A. D'Avolio, A. Ibañez, M. Sciandra, M. Siccardi, D.G. de Requena, S. Bonora, G.D. Perri, J. Chromatogr. B 835 (2006) 127.
[60] R. Mandrioli, M.A. Saracino, S. Ferrari, D. Berardi, E. Kenndler, M.A. Raggi, J. Chromatogr. B 836 (2006) 116.
[61] J. Macek, J. Klíma, P. Ptáček, J. Chromatogr. B 832 (2006) 169.
[62] Y. Hirano, M. Tsunoda, T. Funatsu, K. Imai, J. Chromatogr. B 819 (2005) 41.
[63] T. Togawa, Y. Mukai, K. Ohata, T. Suzuki, S. Tanabe, J. Chromatogr. B 819 (2005) 67.
[64] G.S. Chen, I.B. Lambert, G.R. Douglas, P.A. White, J. Chromatogr. B 824 (2005) 229.
[65] J. Bierau, R. Leen, A.H. van Gennip, H.N. Caron, A.B.P. van Kuilenburg, J. Chromatogr. B 805 (2004) 339.
[66] T. Chikuma, K. Taguchi, M. Yamaguchi, H. Hojo, T. Kato, J. Chromatogr. B 806 (2004) 113.
[67] N. Yasui-Furukori, Y. Inoue, T. Tateishi, J. Chromatogr. B 811 (2004) 153.
[68] T. Uno, N. Yasui-Furukori, T. Takahata, K. Sugawara, T. Tateishi, J. Chromatogr. B 816 (2005) 309.
[69] M. Buffalini, R. Pierleoni, C. Guidi, P. Ceccaroli, R. Saltarelli, L. Vallorani, S. Zeppa, V. Stocchi, J. Chromatogr. B 819 (2005) 307.
[70] A.B.P. van Kuilenburg, L. Zoetekouw, J. Chromatogr. B 820 (2005) 271.
[71] M.N. Khalil, N. Erb, P.N. Khalil, G. Escherich, G.E. Janka-Schaub, J. Chromatogr. B 821 (2005) 105.
[72] Z.Y. Hu, Q. Gong, X.L. Hu, L.Q. Wang, Y.J. Cao, W. Cao, Q. Yu, Z.N. Cheng, J. Chromatogr. B 826 (2005) 238.
[73] K. Oselin, K. Anier, R. Tammb, K. Kallassalu, U. Mäeorg, J. Chromatogr. B 834 (2006) 77.
[74] F. Elbarbry, K. Wilby, J. Alcorn, J. Chromatogr. B 834 (2006) 199.
[75] Y. Singh, A. Misra, K. Misra, Indian J. Chem. Sect. B: Org. Chem. Med. Chem. 41B (2002) 1238.
[76] T. Suga, T. Hirata, Phytochemistry 29 (1990) 2393.
[77] K. Ishihara, H. Hamada, T. Hirata, N. Nakajima, J. Mol. Catal. B: Enzym. 23 (2003) 145.
[78] F. Li, J.N. Cui, X.H. Qian, R. Zhang, Y. Xiao, Chem. Commun. 14 (2005) 1901.
[79] M. Bradford, Anal. Biochem. 72 (1976) 248.
[80] A.L. James, J.D. Perry, C. Jay, D. Monget, J.W. Rasburn, F.K. Gould, Lett. Appl. Microbiol. 33 (2001) 403.