# The Biodegradation of Various Polihydroxy Benzenes with *Pelobacter acidigallici*

**GABRIEL KATONA[1], CECILIA ROMAN[2]*, MIRCEA CHINTOANU[2], ADRIANA GOG[2], GABRIELA PITL[2], MARIUS ROMAN[2], EMIL LUCA[3], CORNELIA MAJDIK[1]**

[1]Babeş-Bolyai University, Faculty of Chemistry and Chemical Engineering, 11 Arany Janos Str., 400028, Cluj-Napoca, Romania
[2]INCDO-INOE Research Institute for Analytical Instrumentation - ICIA, 67 Donath, 400293, Cluj-Napoca, Romania
[3]University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Faculty of Horticulture, 3 Calea Manastur, 400372, Cluj-Napoca, Romania

*The biodegradation of various polihydroxy benzenes with harvested Pelobacter acidigallici cells is described.*

*Keywords: biodegradation, polihydroxy benzenes, Pelobacter acidigallici*

Pyrogallol-phloroglucinol transhydroxylase (TH) from *Pelobacter acidigallici* is one of the important enzymes for the anaerobic degradation of aromatic compounds like gallic acid and various phenols [1,3]. All these compounds are converted into phloroglucinol, which is then reductively dearomatized and degraded to three acetyl-CoA molecules *via* 3-hydroxy-5-oxohexanoate (fig. 1). Such a pathway leads not only to important building blocks but can also be used for ATP synthesis in *Pelobacter acidigallici*.

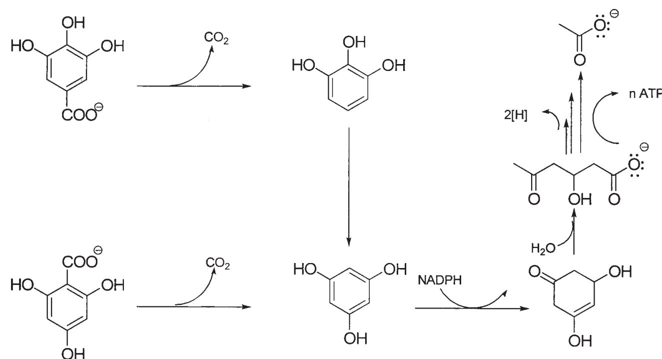


Fig. 1. Biodegradation pathway for different phenols in *Pelobacter acidigallici*

Moreover, this microorganism could be succesfully used for the anaerobic degradation of polihydroxy benzenes from industrial waste water.

Earlier it was showed that TH contains a molybdopterin cofactor (Moco) and iron sulphur clusters [4Fe-4S] and needs 1,2,3,5-tetra-hydroxybenzene as a co-substrate or rather co-catalyst because it is regenerated in the reaction cycle [1,6]. On the basis of these results the reaction was formulated [4,5] as depicted in figure 2.

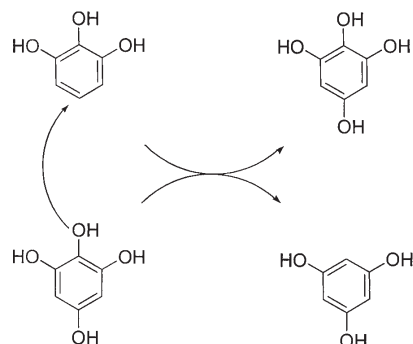


Fig.2. The role of 1,2,3,5-tetra-hydroxybenzene in the mechanism of action of the Mo cofactor containing pyrogallol-phloroglucinol transhydroxylase

From 1,2,3,5-tetra-hydroxybenzene the 2-OH group is transferred to pyrogallol while phloroglucinol and a new molecule of the co-catalyst are produced as also found in other biochemical pathways [7]. It is believed that in Moco-containing hydroxylases which use water as hydroxyl source, the OH-group is first ligated to the molybdenum and then transferred to the substrate, a similar mechanism was discussed also for the TH reaction [8].

A second mechanistic proposal [9,10] described a direct hydroxyl transfer from the co-catalyst 1,2,3,5-tetra-hydroxybenzene to the *ortho*-quinone form of pyrogallol. Here the main function of Moco is the oxidation of pyrogallol to quinone. In this way an enzyme-bound 2,4,6,3',4',5'-hexa-hydroxydiphenylether is generated as intermediate. Fragmentation of this diphenyl ether to phloroglucinol and the ortho-quinone form of tetra-hydroxybenzene is followed by reduction of the latter to regenerate the co-catalyst. In this paper is presented the synthesis of 2,4,6,3',4',5'-hexahydroxy-, as well as 3,4,5,3',4',5'-hexa-hydroxy-diphenyl ether, and describe their interaction with *Pelobacter acidigallici*.

## Experimental part

### *Enzymatic experiments*

*Pelobacter acidigallici* cells were grown and isolated as previously described [6]. The enzyme assay was carried out under anoxic conditions at 30 °C in a discontinuous fashion by HPLC analysis of the products. All ingredients were stored and transferred under argon.

Their concentrations in the assay mixture were as follows: 100 mM potassium phosphate buffer, (*p*H 7.2), 10 μmol pyrogallol, 10 μmol 1,2,3,5-tetra-hydroxybenzene and 10 mg of harvested cells, the total volume of the assay was 1 mL. Samples (20 μL) were withdrawn with a unimetric-pipet and the reaction was terminated by adding 0.1 M $H_3PO_4$ (5 μL) and diluted 10 times with double distilled water followed by the centrifugation of the cells before injection. The withdrawals occurred soon after start of the reaction and then at regular intervals. All experiments were carried out under anaerobic conditions. In case of the reactions in which pyrogallol was incubated with the harvested cells, no reducing agent was required in the assays. The conditions for the HPLC analyses were: on a C18 ec column using as eluent 20 mM HCl in water: acetonitrile (95: 5, v/v) in an isocratic manner at a

* Tel.: (40) 0264593833

1 mL . min$^{-1}$ flow rate. The assays with hexahydroxydiphenyl ethers were similar to the assay described above.

For preparative purposes 10 mL of cell suspension was used and after stopping the reaction with 0.1 M $H_3PO_4$ the mixture was centrifuged to separate the cells. The supernatant was injected in 2 mL portions on a preparative RP-18 column and eluted with 20 mM HCl in water: acetonitrile (95: 5, v/v) in an isocratic manner at a 5 mL . min$^{-1}$ flow rate. Fractions with the same retention time (9 and 12 min. for 1,2,3,5-tetra-hydroxybenzene and 16 and 19 min. for phloroglucinol) of the subsequent separations were combined and the products were obtained after freeze-drying.

*Materials and methods*

Analytical methods

The $^1$H- and $^{13}$C-NMR spectra were recorded on a Bruker spectrometer operating at 400 MHz and 100 MHz, respectively. Mass spectra (MS) were recorded on a VG 7070E mass spectrometer. HPLC analyses were conducted with a Agilent 1200 instrument.

Reagents and solvents

2,4,6-trimethoxybenzaldehyde, 3,4,5-trimethoxyaniline, 3,4,5-trimethoxyphenol, all inorganic reagents and solvents were products of Aldrich or Fluka. All solvents were purified and dried by standard methods as required.

*Synthesis of 3,4,5 trimethoxybromobenzene (**2**)*

To a stirred solution of $CuSO_4 . 5H_2O$ (16.48 g, 66 mmol) and NaBr (10.3 g, 100 mmol) in water (60 mL) was dropwise added a solution of $Na_2SO_3 . 7H_2O$ (8.57 g, 34 mmol) in water (20 mL). For a better precipitation of CuBr, the suspension was cooled to 0°C. After 30 min the supernatant was decanted and the precipitate was washed with cold water (2 . 30 mL). The suspension was rendered clear by adding concentrated HBr (26 mL) and stored under $N_2$. To a half-concentrated HBr water solution (160 mL) 3,4,5-trimethoxyaniline (9.16 g, 50mmol) was added in one portion at room temperature. The suspension was heated to 70°C to obtain a clear solution, which was then cooled to 0°C. Subsequently a solution of $NaNO_2$ (3.45 g, 50 mmol) in water (30 mL) was dropwise added while keeping the temperature below 5°C. The reaction went to completion in 30 min, and then a small portion of urea was added to destroy the non-reacted nitrous acid. To the solution of the diazonium salt, the previously prepared CuBr solution was added at 0°C under argon. The reaction mixture was allowed to warm to room temperature and then was heated to 100°C. The cooled mixture was extracted with diethyl ether (3 . 200 mL), the combined organic layers were washed with HCl solution (20%, 3 . 100 mL), then with water (3 . 100 mL) and finally with KOH solution (20%, 3 . 100 mL). The organic phase was dried over anhydrous $Na_2SO_4$, filtered and the solvent was removed under vacuum. The crude product was purified by column chromatography on silica gel using as eluent dichloromethane yielding pure 3,4,5 trimethoxybromobenzene (9.25 g, 75%).

*Synthesis of 2,4,6 trimethoxyphenol (**3**)*

Into a cooled (ice-bath) solution of 2,4,6-trimethoxybenzaldehyde (5 g, 25.5 mmol) in dry dichloromethane (30 mL), a solution of 3-chloroperoxybenzoic acid (8.6 g, 50 mmol) in dichloromethane (140 mL) was dropwise added during approximately 90 min. The reaction was complete after 3 h at room temperature. The reaction mixture was washed with saturated $NaHCO_3$ solution (3) . 25 mL), dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The crude product was dissolved in methanol (50 mL), cooled to 0 °C and then KOH solution (5 g in 25 mL water) was added within 20 min. The reaction was complete in 45 min at 0 °C. Then the pH of the solution was adjusted to 2 by adding HCl solution (2 M). The reaction mixture was extracted with diethyl ether (3 . 200 mL), the etheric phase was washed with water (3 . 100 mL), the organic layer was dried over anhydrous $Na_2SO_4$. The solvent was removed by distillation under reduced pressure and the crude product was purified by column chromatography on silica gel using as eluent dichloromethane: acetone mixture (95:5, v/v). The pure 2,4,6 trimethoxyphenol (2.34 g, 50%) was crystallized in 2-3 days.

*Preparation of hexa-methoxydiphenyl ethers **4** and **5***

2,4,6-trimethoxyphenol (**3**) or 3,4,5-trimethoxyphenol (2.46 g, 13.4 mmol), 1-bromo-3,4,5-trimethoxybenzene (**2**) (6.62 g, 26.8 mmol) and CuO (4.3 g, 30 mmol) were refluxed in 2,4,6-collidine (35 mL) for 63 h under argon. The cooled reaction mixture was treated with half-concentrated HCl (conc. HCl:water 1:1,150 mL) and extracted with diethyl ether (4 . 200 mL). The combined organic layers were washed with half-concentrated HCl (500 mL), then with KOH solution (20%, 750 mL) and finally with water. The dried organic layer was concentrated under reduced pressure and the crude product was purified by column chromatography on silica gel (dichloromethane: acetone (90:10, v/v) to yield:

2,4,6,3',4',5'- Hexa-methoxydiphenyl ether (**4**), amorphous white powder. Yield 41%. HRMS M+1 found (M+1 calculated for $C_{18}H_{23}O_7$): 351.1450 (351.1444); $^1$H NMR: $\delta(CD_3OD)$ = 3.75 (s, 6H), 3.78 (s, 3H), 3.79 (s, 6H), 3.84 (s, 3H), 6.12 (s, 2H), 6.23 (s, 2H); $^{13}$C NMR: $\delta(CD_3OD)$ = 55.5, 55.9, 56.3, 60.9, 91.8, 92.2, 125.9, 132.6, 153.6, 153.9, 155.3, 157.6.

3,4,5, 3',4',5'- Hexa-methoxydiphenyl ether (**5**), amorphous white powder. Yield 48%. HRMS M+1 found (M+1 calculated for $C_{18}H_{23}O_7$): 351.1446 (351.1444); $^1$H-NMR: $\delta(CD_3OD)$ = 3.67 (s, 12H), 3.81 (s, 6H), 6.28 (s, 4H); $^{13}$C-NMR: $\delta(CD_3OD)$ = 56.2, 61.0, 96.3, 134, 153.4, 153.9.

*Preparation of 1,2,3,5-tetra-hydroxybenzene (**1**) and of the hexahydroxydiphenyl ether **6** and **7***

To a solution of 3,4,5-trimethoxyphenol and hexamethoxydiphenyl ether **4** or **5** (1.88 mmol) in dichloromethane (10 mL) was added 1 M boron tribromide solution in dichloromethane (18.8 mL) at -80°C under argon. After stirring for 1 h at -80°C the reaction mixture was left to warm up to room temperature in 1h. After 10 h the solution was cooled to 0°C and water (5 mL) was added. After removal of the dichloromethane under reduced pressure the remaining mixture was extracted with ethyl acetate (3x50 mL). The combined organic phases were dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure. The crude product was treated with water (20 mL) and the suspension was centrifuged. The supernatant was decanted and purged with argon. After that 2 mL of the mixture was purified on a preparative RP-18 column using as eluent 20 mM HCl in water: acetonitrile (95: 5, v/v) at a 5 mL . min$^{-1}$ flow rate. Fractions with the retention time between 9 and 14 min. for (**1**), 30 and 45 min. for (**6**) and 20 and 35 min. for (**7**) were collected and the pure products were obtained after freeze-drying.

1,2,3,5-tetra-hydroxybenzene (**1**): Yield 52%. HRMS M+1 found (M+1 calculated for $C_6H_6O_4$): 142.0266 (142.0247); $^1$H-NMR: $\delta(D_2O)$ = 5.93 (s, 2H); $^{13}$C-NMR: $\delta(D_2O)$ = 95.5, 125.4, 145.3, 146.4.

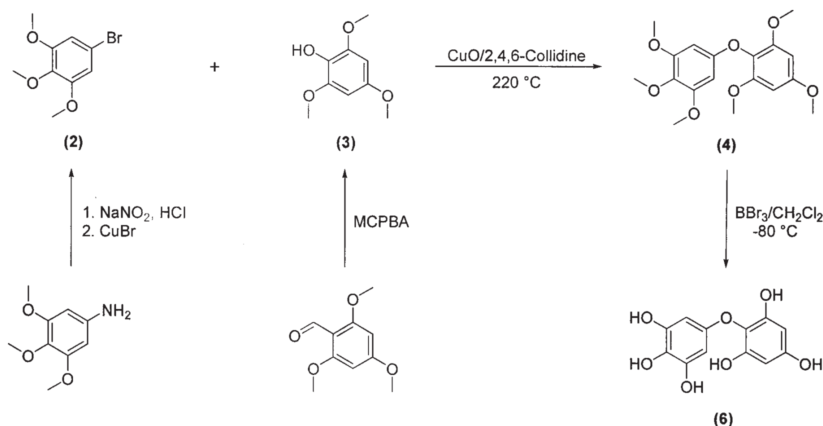


Fig.3. Synthesis of the proposed intermediate, 3,4,5,2',4',6'-hexa-hydroxydiphenyl ether.

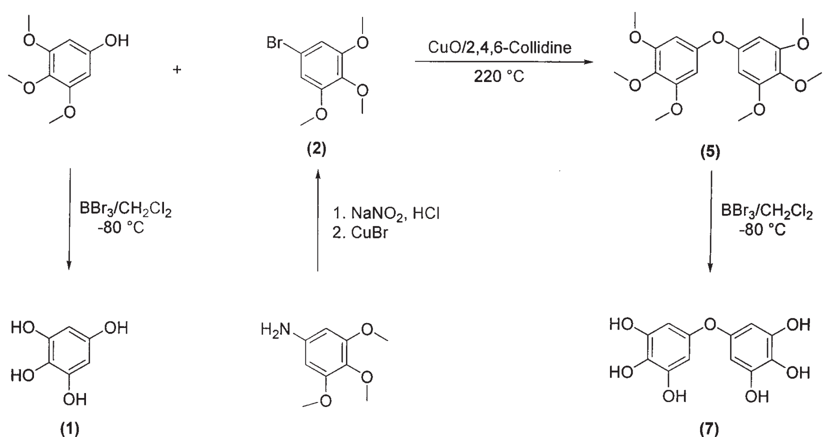


Fig.4. Synthesis of 1,2,3,5-tetra-hydroxybenzene and of the symmetrical 3,4,5,3',4',5'-hexa-hydroxydiphenyl ether

2,4,6,3',4',5'- Hexa-hydroxydiphenyl ether (**6**), amorphous white powder. Yield 58%. HRMS M+1 found (M+1 calculated for $C_{12}H_{11}O_7$): 267.0505 (267.0505); $^1$H-NMR: $\delta(D_2O)$ = 5.92 (s, 2H), 5.98 (s, 2H); $^{13}$C-NMR: $\delta(D_2O)$ = 94.2, 94.8, 123.9, 127.1, 146, 151.1, 152.1, 154.6.

3,4,5,3',4',5'- Hexa-hydroxydiphenyl ether (**7**). Yield 78%. HRMS M+1 found (M+1 calculated for $C_{12}H_{11}O_7$): 267.0505 (267.0501); $^1$H-NMR: $\delta(D_2O)$ = 5.97 (s, 4 H); $^{13}$C-NMR: $\delta(D_2O)$ = 97.7, 128.3, 146.1, 150.5.

## Results and discussions

*Synthesis of hexa-hydroxydiphenyl ethers*

2,4,6,3',4',5'- and 3,4,5,3',4',5'-Hexa-hydroxydiphenyl ethers were found as components of the ethyl acetate soluble mixture of poly-hydroxyphenyl esters occurring in various kinds of algae like *Bifurcaria bifurcate*, *Caprophyllum maschalocarpumas*, etc. [11,12].Only the hexa-acetylated form of the diphenyl ether was isolated, the unprotected compound could not be obtained in pure form due to its oxygen sensitivity. Both hexa-hydroxy-diphenyl ethers were previously synthesized from the corresponding hexa-methoxydiphenyl ethers using boron tribromide as demethylation agent,[13] but these were not isolated and were transformed to dibenzofurans [14]. The syntheses of the hexa-hydroxydiphenyl ethers and 1,2,3,5-tetra-hydroxybenzene are presented in figure 3 and 4.

*Interaction of the Hexa-hydroxydiphenylethers with harvested Pelobacter acidigallici cells*

Since there is no measurable difference in the UV-absorption of the investigated polihydroxypehnols the kinetics of their biodegradation was performed with HPLC analysis. Under the conditions used, the retention times for 1,2,3,5-tetra-hydroxybenzene, 2,4,6,3',4',5'-hexa-hydroxydiphenyl ether, phloroglucinol, pyrogallol, and 3,4,5,3',4',5'-hexa-hydroxydiphenyl ether were approximately 4.5, 7.1, 7.9, 8.7 and 19.3 min , respectively. In some cases small deviations from these values were observed.

When the harvested cells were incubated with pyrogallol only, a moderate reaction rate was detected. In the presence of 1,2,3,5-tetra-hydroxybenzene phloroglucinol was produced with higher rate. Even after longer incubation (50 min) at 30°C the concentration of the 1,2,3,5-tetra-hydroxy benzene was only slightly since it was regenerated in the reaction cycle. This observation is in good agreement with the proposed role of 1,2,3,5-tetra-hydroxybenzene as co-catalyst. Based on the areas under the HPLC traces and the amounts of the pyrogallol (10 μmol) and harvested cells (10 mg) used in the reaction the activity can be estimated to be 0.2 μmol/min/mgcell. Since some oxidation of the extremely oxygen sensitive poly-hydroxyphenols was unavoidable, the limit of error is estimated to be about 10%. In order to investigate whether the *Pelobacter acidigallici* cells will accept 2,4,6,3',4'5'-hexa-hydroxydiphenyl ether as substrate, the latter and pyrogallol were added to the reaction mixture. Under these conditions, as predicted phloroglucinol and 1,2,3,5-tetra-hydroxybenzene were formed. After 60 min reaction time, about 90% of the pyrogallol and 2,4,6,3',4,',5'-hexa-hydroxydiphenyl ether was converted into phloroglucinol. On the bases of the areas under the HPLC traces and the amounts of the pyrogallol, hexa-hydroxydiphenyl ether and enzyme it is estimated that in the enzymatic reaction about 2 μmol 2,4,6,3',4',5'-hexa-hydroxydiphenyl ether and pyrogallol was consumed per min. In a further experiment 2,4,6,3',4',5'-hexa-hydroxydiphenyl ether was incubated with the harvested cells. In this case no reaction occurred,

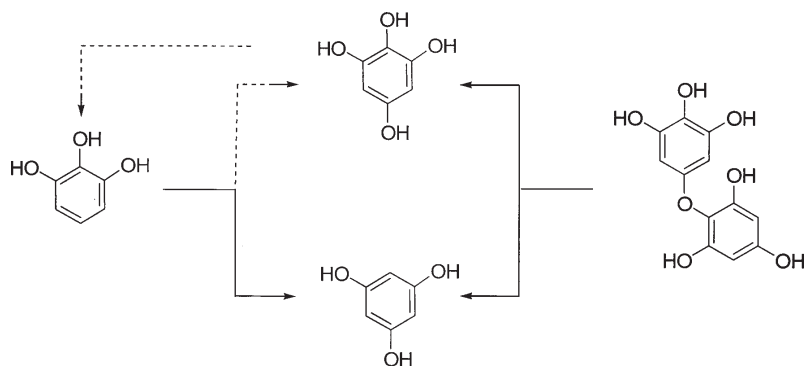


Fig. 5. Proposed path for the biotransformation of of the unsymmetrical hexahydroxy diphenyl ether.

but when an equimolar amount of glutathione was introduced into the reaction mixture, phloroglucinol and 1,2,3,5-tetra-hydroxybenzene were formed. These results show that a reducing agent is necessary to keep the reaction going, since according to figure 5 the cleavage of the hexa-hydroxydiphenyl ether leads, in addition to phloroglucinol, to the enzyme-bound *para*-quinone form of 1,2,3,5-tetra-hydroxybenzene. In the absence of pyrogallol glutathione can reduce the enzyme-bound quinone to provide the co-catalyst.

The symmetrical 3,4,5,3',4',5'-hexa-hydroxydiphenyl ether could also play the role of a substrate for *Pelobacter acidigallici*. Its retention time was however completely different, from that of its unsymmetrical counterpart, namely around 18 min. 3,4,5,3',4',5'-Hexa-hydroxydiphenyl ether alone was completely inert with transhydroxylase and no detectable amount of phloroglucinol was found even when an equimolar amount of glutathione was added to the reaction mixture. Addition of pyrogallol initiated a reaction, which produced phloroglucinol. After 4 h, more phloroglucinol was formed and the amount of pyrogallol was reduced by about 90% (fig. 5), while only a relative small portion of the 3,4,5,3',4',5'-hexa-hydroxydiphenyl ether was consumed. The reactions illustrated in figure 4 and 5. were performed also in preparative scale, the isolated products showed the same analytical data as reference 1,2,3,5-tetra-hydroxybenzene and phloroglucinol. These results suggest that the symmetrical hexa-hydroxydiphenyl ether can also intrude into the active site of the enzyme and is cleaved into pyrogallol and the *para*-quinone form of 1,2,3,5-tetra-hydroxybenzene, although much slower than its unsymmetrical counterpart.

## Conclusions

In conclusion, the biodegradation of trihydroxy benzenes and hexahydroxy-diphenyl ethers with water suspended *Pelobacter acidigallici* cells is reliable and is a good alternative for the purification of waste waters containing polihydroxy phenols.

*Acknowledgments:: The financial support from the Romanian Ministry of Education and Research (PNCD II Project Nr. 31-016/2007, DEFENOL) is gratefully acknowledged.*

## References

1. SCHINK, B., PFENNIG, N., Arch. Microbiol., **133**, 1982, p.195.
2. BRUNE, A., SCHINK, B., J. Bacteriol., **172**, 1990, p. 1070
3. BRUNE, A., SCHINK, B., Arch. Microbiol., **157**, 1992, p. 417
4. BRUNE, A., SCHNELL, S., SCHINK, B. Appl. Evironmental Microbiol., **58**, 1992, p. 1861
5. REICHENBECHER, W., SCHINK, B. Biochim. Biophys. Acta, **1430**, 1999, p. 245
6. REICHENBECHER, W., BRUNE, A., SCHINK,. B. Biochim. Biophys. Acta, **1204**, 1994, p. 217
7. LEHNINGER, A.L., NELSON, D.L., COX, M.M., Principles of Biochemistry, 2.Edition, Worth Publisher, New York, 1993, p.412
8. HILLE, R. Chem. Rev., **96**, 1996, p. 2757
9. . HILLE, R., RÉTEY, J., BARTLEWSKI-HOF, U., REICHENBECHER, W., SCHINK, B., FEMS Microbiol. Rev., **22**, 1999, p. 489
10. BOLL, M., SCHINK, B., MESSERSCHMIDT, A.,. KRONECK, P.M.H., Biol.Chem., **386**, 2005, p. 999
11.GLOMBITZA, K.W., RÖSNER, H.U., Phytochemistry, **13**, 1974, p. 1245
12. GLOMBITZA, K.W., LI, S.M., Phytochemistry, **30**, 1991, p. 2741
13. MCOMIE, J.F.W., WATTS, M.L., WEST, D.E., Tetrahedron, **24**, 1968, p. 2289
14. GLOMBITZA, K.W., WÖWLER-RIECK, U., Liebigs Ann., 1988, p. 261

Manuscript received: 26.11.2009