JSCSEN 73(10)937-1019(2008)

Journal of the Serbian Lectronic Chemical Society

VOLUME 73

NO 10

BELGRADE 2008

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Published by the Serbian Chemical Society	
Karnegijeva 4/III. 11000 Belgrade. Serbia	

Published by the Serbian Chemical Society Karnegijeva 4/III, 11000 Belgrade, Serbia Printed by the Faculty of Technology and Metallurgy Karnegijeva 4, P.O. Box 35-03, 11120 Belgrade, Serbia





J. Serb. Chem. Soc. 73 (10) 937–943 (2008) JSCS–3774 JSCS@tmf.bg.ac.yu • www.shd.org.rs/JSCS UDC 547–304.4+546.33–36:615.281/.282 Original scientific paper

Synthesis and antimicrobial, antifungal and anthelmintic activities of 3*H*-1,5-benzodiazepine derivatives

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(Received 1 August 2007, revised 14 January 2008)

Abstract: The diazonium salt of 4-amino-1-methyl-3-propyl-1*H*-pyrazole-5-carboxamide in the presence of sodium hydroxide was condensed with different β -diketones/ β -ketoesters, **3a–e**, to obtain new β -diketones/ β -ketoesters, **4a–e**. The β -diketones/ β -ketoesters **4a–e** were condensed with *o*-phenylenediamine (*o*-PDA) in presence of *p*-toluenesulfonic acid/SiO₂ to give biologically active 3*H*-1,5--benzodiazepines, **5a–e**. All the newly synthesized compounds were characterized by elemental analysis and spectral studies. The compounds **5a–e** were screened for their antimicrobial, antifungal and anthelmintic activities.

Keywords: 1,5-benzodiazepines; β -diketones/ β -ketoesters; substituted pyrazole; *p*-toluenesulfonic acid.

INTRODUCTION

Benzodiazepines have attracted attention as an important class of heterocyclic compounds in the field of drugs and pharmaceuticals. These compounds are widely used as anticonvulsant, anti-anxiety, analgesic, sedative, antidepressive and hypnotic agents,¹ as well as anti-inflammatory agents.² Other than their biological importance, benzodiazepines derivatives are also commercially used as dyes for acrylic fibers.³ Moreover, 1,5-benzodiazepines derivatives are valuable synthons that can be used in the preparation of other fused ring compounds, such as triazolo-, oxadiazolo-, oxazino- or furano-benzodiazepines.⁴

Research in this area is still very active and is directed towards the synthesis of compounds with enhanced pharmacological activity. Generally, these compounds are synthesized by the condensation of *o*-phenylenediamines with α , β -unsaturated carbonyl compounds,⁵ β -haloketones or ketones.⁶ A variety of reagents, such as BF₃-etherate, NaBH₄, polyphosphoric acid, or SiO₂, MgO//POCl₃, Yb(OTf)₃, Sc(OTf)₃, Al₂O₃/P₂O₅, or AcOH under microwave and in ionic liquids⁷ have been utilized for the condensation reaction. Most recently, this condensation has also been reported to proceed in the presence of CAN, bromodimethylsulfonium bromide, organic acids and AgNO₃.⁸

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doi: 10.2298/JSC0810937K

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However, all these methods have disadvantages, such as extreme reaction conditions and also several side-reactions. Surface-mediated solid phase reactions are of growing interest⁹ because of their ease of execution and work-up, mild reaction conditions, rate of reaction, selectivity, high yields, lack of solvents and low cost in comparison to their homogeneous counter parts. Previously, efforts were made to explore the utility of surface-mediated reaction.^{10–12}

Herein, a new method for the preparation of 1,5-benzodiazepine derivatives with β -diketones and β -ketoesters is reported. It was found that a mixture of *p*-to-luenesulfonic acid/SiO₂ under solvent-free conditions was capable of producing high yields of 1,5-benzodiazepines **5a–e**, by condensation of *o*-phenylenediamine with dicarbonyl compounds **4a–e** under mild conditions.

Our interest laid in the synthesis the pyrazole moiety containing 1,5-benzodiazepines, as pyrazoles are known to have significant pharmacological properties. They exhibit antimicrobial,¹³ anti-inflammatory,¹⁴ antiviral¹⁵ and pesticidal activity.¹⁶ Substituted pyrazoles are useful for their cardiovascular,¹⁷ antitumor¹⁸ and hypolipemic activity. It is interesting to note that pyrazoles are reported as well known pharmaceuticals.^{19–21}

RESULTS AND DISCUSSION

Diazonium salt of 4-amino-1-methyl-3-propyl-1*H*-pyrazole-5-carboxamide was formed in the presence NaNO₂/HCl at 0–5.0 °C. Diazonium salt was condensed with different β -diketones/ β -ketoesters in the presence of sodium hydroxide (Scheme 1). The newly synthesized β -diketones/ β -ketoesters **4a**–e were condensed with *o*-phenylenediamine (*o*-PDA) in the presence of *p*-toluenesulfonic acid, to obtain 3*H*-1,5-benzodiazepines **5a–e** (Scheme 2).



Scheme 1.

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Spectral Analysis

4-[(2,4-Dimethyl-3H-1,5-benzodiazepin-3-yl)azo]-1-methyl-3-propyl-1H-pyrazole-5-carboxamide (**5a**): Yield: 88 %; m.p. 215 °C; Anal. Calcd. for C₁₉H₂₃N₇O: C, 62.46; H, 6.30; N, 26.84. Found: C, 62.32; H, 6.20; N, 26.74. IR (KBr, cm⁻¹): 3400, 3450, 3050, 2930, 1680, 1585, 1435. ¹H-NMR (300 MHz, CDCl₃, δ, ppm): 1.03 (3H, *t*, CH₃, *J* = 7.86 Hz), 1.66 (2H, *m*, CH₂, *J* = 8.25 Hz), 2.15 (6H, *s*, CH₃), 2.55 (2H, *t*, CH₂, *J* = 7.89 Hz), 3.35 (1H, *s*, CH=), 3.80 (3H, *s*, N–CH₃), 7.55–7.80 (4H, *m*, Ar–H, *J* = 7.55 Hz), 8.1 (2H, *s*, CONH₂). ¹³C-NMR (75 MHz, CDCl₃, δ, ppm): 15.80 (CH₃–C=N), 18.71 (CH₃), 28.95 (CH₂), 40.38 (CH₂), 40.58 (N–CH₃), 134.65 (C=N), 140–144.7 (Ar–C), 169.79 (CONH₂). LCMS: 366 (M + H⁺).

4-[(2,4-Diphenyl-3H-1,5-benzodiazepin-3-yl)azo]-1-methyl-3-propyl-1H-pyrazole-5-carboxamide (**5b**): Yield: 76.6 %; m.p. 245 °C; Anal. Calcd. for C₂₉H₂₇N₇O: C, 71.16; H, 5.72; N, 20.04. Found: C, 71.00; H, 5.68; N, 20.04. IR (KBr, cm⁻¹): 3405, 3520, 3045, 2925, 1680, 1620, 1448. ¹H-NMR (300 MHz, CDCl₃, δ, ppm): 1.03 (3H, t, CH₃, J = 7.86 Hz), 1.66 (2H, m, CH₂, J = 8.25 Hz), 2.53 (2H, t, CH₂, J = 7.89 Hz), 3.42 (1H, s, CH=), 3.80 (3H, s, N–CH₃), 7.62–7.95 (14H, m, Ar–H, J = 7.55 Hz), 8.20 (2H, s, CONH₂). ¹³C-NMR (75 MHz, CDCl₃, δ, ppm): 15.68 (CH₃), 27.57 (CH₂), 39.37 (N–CH₃), 41.37 (CH₂), 135.00–143.67 (Ar–C), 166.23 (C=N), 166.87 (CONH₂). LCMS: 490 (M + H⁺).

4-[(2-Methyl-4-phenyl-3H-1,5-benzodiazepin-3-yl)azo]-1-methyl-3-propyl-1H--pyrazole-5-carboxamide (5c): Yield: 82 %, m.p.256 °C; Anal. Calcd. for C₂₄H₂₅N₇O: C, 67.45; H, 5.89; N, 22.70. Found: C, 67.44; H, 5.90; N, 22.73. IR (KBr, cm⁻¹): 3415, 3485, 3030, 2910, 1685, 1435. ¹H-NMR (300 MHz, CDCl₃, δ, ppm): 1.02 (3H, *t*, CH₃, *J* = 7.86 Hz), 1.65 (2H, *m*, CH₂, *J* = 8.25 Hz), 2.20 (3H, *s*, CH₃), 2.54 (2H, *t*, CH₂, *J* = 7.89 Hz), 3.40 (1H, *s*, CH=), 3.86 (3H, *s*, N–CH₃), 7.50– -7.80 (4H, *m*, Ar–H, *J* = 7.55 Hz), 8.20 (2H, *s*, CONH₂), 10.25 (1H, *s*, N–H). ¹³C-NMR (75 MHz, CDCl₃, δ, ppm): 15.77 (CH₃–C = N), 18.52 (CH₃), 28.61 (CH₂), 38.75 (N–CH₃), 40.04 (CH₂), 140.00–147.45 (Ar–C), 159.23 (–C–), 164.70 (C=N), 167.25 (CONH₂). LCMS: 428 (M + H⁺).

4-[(2,3-Dihydro-4-methyl-2-oxo-1H-1,5-benzodiazepin-3-yl)azo]-1-methyl-3--propyl-1H-pyrazole-5-carboxamide (5d): Yield: 77 %; m.p. 230 °C; Anal. Calcd. for C₁₈H₂₁N₇O₂: C, 58.85; H, 5.99; N, 26.70. Found: C, 58.83; H, 5.97; N, 26.67. IR (KBr, cm⁻¹): 3420, 3480, 3040, 2915, 1685, 1430. ¹H-NMR (300 MHz; CDCl₃; δ, ppm): 1.02 (3H, *t*, CH₃, *J* = 7.86 Hz) , 1.65 (2H, *m*, CH₂, *J* = 8.25 Hz), 2.20 (3H, *s*, CH₃), 2.54 (2H, *t*, CH₂, *J* = 7.89 Hz), 3.4 (1H, *s*, CH=), 3.85 (3H, *s*, N–CH₃), 7.50–7.80 (4H, *m*, Ar–H, *J* = 7.55 Hz), 8.2 (2H, *s*, CONH₂), 10.25 (1H, *s*, N–H). ¹³C-NMR (75 MHz, CDCl₃, δ, ppm): 15.74 (CH₃–C=N), 18.54 (CH₃), 28.65 (CH₂), 38.58 (N–CH₃), 40.07 (CH₂), 140.00–147.79 (Ar–C), 159.56 (–C–), 164.77 (C=N), 167.20 (CONH₂). LCMS: 368 (M + H⁺).

4-[(2,3,4,5-tetrahydro-2,4-dioxo-1H-1,5-benzodiazepin-3-yl)azo]-1-methyl-3--propyl-1H-pyrazole-5-carboxamide (5e): Yield: 51.5 %; m.p. 205 °C; Anal. Calcd. for C₁₇H₁₉N₇O₃: C, 55.28; H, 5.42; N, 26.55. Found: C, 55.24; H, 5.32; N, 26.45. IR (KBr, cm⁻¹) 3430, 3500, 3020, 2930, 1685, 1595, 1437. ¹H-NMR (300 MHz, CDCl₃, δ, ppm): 1.04 (3H, *t*, CH₃, *J* = 7.86 Hz), 1.66 (2H, *m*, CH₂, *J* = 8.25Hz), 2.55 (2H, *t*, CH₂, *J* = 7.89 Hz), 3.42 (1H, *s*, CH=), 3.80 (3H, *s*, N–CH₃), 7.5–7.8 (4H, *m*, Ar–H, *J* = = 7.55 Hz), 8.15 (2H, *s*, CONH₂), 10.20 (2H, *s*, N–H). ¹³C-NMR (75 MHz, CDCl₃, δ, ppm): 16.78 (CH₃), 29.92 (CH₂), 41.32 (CH₂), 37.59 (N–CH₃), 138–145 (Ar–C), 163.74 (C=N), 166.28 (CONH₂). LCMS: 370 (M + H⁺).

Antimicrobial and anthelmintic activities of compounds 5a-e

The newly synthesized diazepine compounds were screened for antibacterial activity against *Staphylococcus aureus* and *Klebsiella pneumoniae* and antifungal activity against *Aspergillus niger* and *Candida albicans* by the cup-plate method.^{22,23} Crofloxin and ciclopiroxolamine were used as standards for comparison of the antibacterial and antifungal activities, respectively. The results indicate that these compounds were active against all the four organisms. The anthelmintic activity was tested on earth worms *Pherituma posthuma*, by a technique described by Bagavant *et al.*²⁴ with modification. Piperazine citrate was used as the standard drug. The results of the antimicrobial and anthelmintic activity are re-

ported in Table I. Compound **5c** exhibited greater antimicrobial and antifungal activities than the standard drugs, whereas compounds **5d** and **5e** showed significant anthelmintic activity.

EXPERIMENTAL

All the melting points were determined in open capillary tubes and are uncorrected. The IR spectra were recorded on a Nicolet-Megna-FT-IR-550 spectrometer in KBr pellets. The ¹H- and ¹³C-NMR spectra were run on a model DRX 300 instrument at 300.13 and 75 MHz, respectively, in CDCl₃ using TMS as an internal standard. The mass spectra were obtained on an LCMS instrument. The purity of the newly synthesized compounds was checked by TLC. Satisfactory C, H, N analyses were obtained for all the compounds.

Compd.	Antibacteria inhib	l activity zone of ition, mm	Antifunga of inhi	l activity zone bition, mm	Anthelmintic activity, min	
	A. aurens	K. pneumoniae	A. niger	C. albicans	Paralysis	Death
5a	11	10	15	18	95	90
5b	10	10	16	17	90	120
5c	22	24	25	28	105	112
5d	12	15	15	11	102	124
5e	14	9	10	19	105	129
Std.	24	26	22	24	100	125

TABLE I. Antimicrobial and anthelmintic activities of compounds 5a-e

Synthesis of β -diketones and β -ketoesters (4*a*–*e*)

The diazonium salt was condensed with β -diketones and β -ketoesters **3a**–e (0.010 M) in the presence of NaOH by continuously stirring the reaction mixture for 6–8 h at 60 °C. The progress of the reaction was monitored periodically by TLC. After completion of the reaction, the reaction mass was poured into ice, acidified and extracted into chloroform (2×50 ml). The solvent was evaporated and the residue dried under vacuum. After crystallization using petroleum ether and ethyl acetate, the purity of the compound was checked through TLC using 7:2:1 benzene:ethanol:ammonia as the mobile phase.

Synthesis of 3H-1,5-benzodiazepines (5a-e)

The 1,3-diketones/1,3-ketoesters **4a**–e (0.010 M) and *p*-toluenesulfonic acid/SiO₂ (*p*-toluenesulfonic acid (1.0 g) and silica gel (1.0 g) in acetone were stirred for 0.5 h on a magnetic stirrer and then the acetone was removed under vacuum) were mixed in a mortar for 10 min. To this mixture in a conical flask, *o*-phenylenediamine (0.010 M) was added. The reaction mixture was heated on a water bath at 60 °C for 30 min. The reaction mixture was washed with dichloromethane (2×50 ml), dried over Na₂SO₄ and the solvent evaporated to give the crude products. The crude products were washed with ether to remove the unreacted dicarbonyl compounds and then crystallized from petroleum ether:ethyl acetate (1:1). The purity of the compounds was checked by TLC using 7:2:1 benzene:ethanol:ammonia as the mobile phase.

Acknowledgement. Authors are thankful to the Head of Department of Chemistry, University of Rajasthan, Jaipur, for providing the laboratory facilities. The authors are also grateful to the Central Drug Research Institute, Lucknow, for providing the spectral data. Rajesh Kumar is thankful to the UGC for the award of a Senior Research Fellowship.

ИЗВОД

СИНТЕЗА И АНТИМИКРОБНА, АНТИФУНГАЛНА И АНТИХЕЛМИТИНСКА АКТИВНОСТ 3*H*-1,5-БЕНЗОДИАЗЕПИНСКИХ ДЕРИВАТА

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Диазонијум со 4-амино-1-метил-3-пропил-1*H*-пиразол-5-карбоксамида кондензована је са различитим β -дикетонима/ β -кетоестрима **3а**–е у присуству натријум-хидроксида дајући нове β -дикетоне/ β -кетоестре **4а**–е. Ови β -дикетони/ β -кетоестри **4а**–е кондензовани су са o-фенилендиамином (o-PDA) у присуству p-толуенсулфонске киселине/SiO₂ при чему се граде биолошки активни 3*H*-1,5-бензодиазепини **5а**–е. Новодобијена једињења окарактерисана су на основу елементалних анализа и спектралних података. Једињења **5а**–е су тестирана на антимикробну, антифунгалну и антихелмитинску активност.

(Примљено 1. августа 2007, ревидирано 14. јануара 2008)

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J. Serb. Chem. Soc. 73 (10) 945–950 (2008) JSCS–3775 JSCS@tmf.bg.ac.yu • www.shd.org.rs/JSCS UDC 547–327+66.095.253:54–732+537.5:544.015.4 Short communication

SHORT COMMUNICATION Benzylation of N-phenyl-2-phenylacetamide under microwave irradiation

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(Received 5 July, revised 21 November 2007)

Abstract: N-Phenyl-2-phenylacetamide was alkylated with benzyl chloride in the presence of powdered potassium hydroxide under microwave irradiation in a solvent-free system. The reactions were also performed in the presence of phase-transfer catalysts. The formation of N-, O- and C-products of alkylation was followed by gas chromatography. The N-product was found to be the main product under microwave irradiation. The O-product was obtained in higher yields when an excess of base and benzyl chloride was used.

Keywords: alkylation; amides; phenylacetamides; phase-transfer conditions; microwave irradiation.

INTRODUCTION

N-Substituted 2-phenylacetamides are very interesting compounds because of their structural similarity to the lateral chain of natural benzylpenicillin.¹ Selective *O*-alkylation of *N*-substituted 2-phenylacetamides is of practical importance in penicillin chemistry, not only for use in chemical transformations of natural benzylpenicillin into 6-aminopenicillanic acid, but also because of the possibility of direct transformation of the imino ether into a new semi-synthetic penicillin. On the other hand, *N*-alkylation yields *N*,*N*-disubstituted 2-phenylacetamides, which are important intermediates in the production of herbicides and tertiary amines.²

Alkylation of *N*-substituted 2-phenylacetamides under neutral and basic conditions has been reviewed recently.² Alkylation of *N*-phenyl-2-phenylacetamide (1, Scheme 1) has also been studied.^{3–6} Work *et al.*³ showed that when 1 is alkylated with benzyl chloride in the presence of sodium amide, only the *C*-product is formed. Torosyan *et al.*⁴ alkylated 1 with benzyl chloride under phase-transfer conditions and obtained only the *N*-product in 48 % yield. Benzylation of

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doi: 10.2298/JSC0810945M

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N-phenyl-2-phenylacetamide with benzyl chloride in the presence of powdered KOH was performed in the presence of various phase-transfer catalysts, solvents and temperatures.⁵ The *N*-product was found to be the main product in most of the employed systems. In an excess of potassium hydroxide and benzyl chloride at reflux temperature in toluene, an almost quantitative yield of the *N*-product was obtained after 4 h of heating. When **1** was alkylated with ethyl bromide under phase-transfer conditions, in addition to the *N*-product, the *O*-product was also detected.⁶



Scheme 1. Alkylation of *N*-phenyl-2-phenylacetamide (1) with benzyl chloride in the presence of KOH under microwave irradiation. Product **2** is the *O*-product (benzyl ester of phenylacetic acid, PAA), product **3** is the *N*-product (*N*-benzyl-*N*-phenyl-2-phenylacetamide) and product **4** is the *C*-product (*N*-phenyl-2,3-diphenylpropanamide) of alkylation.

Microwave irradiation has become an important method in organic synthesis which is applicable to a wide range of reactions with short reaction times and high yields. Reactions in the absence of solvent (solvent-free synthesis) under microwave irradiation also offer several advantages. The absence of solvent reduces the risk of explosion and simplifies the work-up procedure.⁷ Such procedures are often examples of green chemistry.

Fast solvent-free alkylation of amides and lactams under microwave irradiation was reported.⁸ Under PTC/OH conditions in a very short time (2.5 min), *N*-alkylation was achieved in high yield but phenylacetamides were not used. Conveniently, the author used amides which can be easily *N*-alkylated, such as *N*-methylacetamide, *N*-phenylacetamide, *N*-phenylbenzamide, caprolactam and valerolactam. To the best of our knowledge, no other study of other reaction products of alkylation of amides under microwave irradiation has hitherto been performed.

In our study of the alkylation of *N*-substituted 2-phenylacetamides,² in this paper, the alkylation of *N*-phenyl-2-phenylacetamide with benzyl chloride under microwave irradiation is now reported. The reactions were performed in a solvent-free system with and without phase-transfer catalysts.

EXPERIMENTAL

Materials

The starting *N*-phenyl-2-phenylacetamide (1) and the expected alkylation products, *i.e.*, the O-(2), *N*-(3) and *C*-product (4), were prepared as follows.

The starting amide (1) was obtained by the reaction of phenylacetyl chloride and aniline.⁹ ¹H-NMR (CDCl₃, δ, ppm): 3.70 (2H, s, Ph–CH₂), 7.0–7.5 (10H, m, ArH); m.p. 114–116 °C.

N-Benzylaniline was obtained by the alkylation of aniline with benzyl chloride in the presence of KOH and tetrabutylammonium hydrogensulfate by mixing 0.10 mol aniline, 0.10 mol KOH, 1.25 mmol tetrabutylammonium hydrogensulfate and 11.2 ml of water. Then 0.10 mol benzyl chloride was added dropwise to the mixture heated on a boiling water bath. After 5 h heating and stirring, the reaction mixture was cooled to room temperature and water was added. The layers were separated and the water layer was extracted with diethyl ether. The organic layers were combined and dried over anhydrous sodium sulfate. The product was isolated by distillation. ¹H-NMR (CDCl₃, δ , ppm): 3.75 (1H, *s*, NH), 4.17 (2H, *s*, CH₂), 6.60 (5H, *q*, Ph–N), 7.23 (5H, *s*, Ph–CH₂); b.p. 112–117 °C (0.40 mbar).

The benzyl ester of PAA (2) was prepared from benzyl chloride and phenylacetic acid, in the presence of 40 % sodium hydroxide and tetrabutylammonium hydrogensulfate.⁹ ¹H-NMR (CDCl₃, δ , ppm): 3.62 (2H, *s*, CH₂–Ph), 5.10 (2H, *d*, O–CH₂), 7,27 (10H, *s*, 2×Ph); b.p. 169– –171 °C (3.0 mbar).

N-Benzyl-*N*-phenyl-2-phenylacetamide (**3**) was synthesized by the same method as for **1** but from phenylacetyl chloride and *N*-benzylaniline. ¹H-NMR (CDCl₃, δ , ppm): 3.70 (2H, *s*, CH₂–CO), 4.90 (2H, *s*, N–CH₂), 6.70–7.30 (15H, *m*, 3×ArH); m.p. 86–88 °C.

N-Phenyl-2,3-diphenylpropanamide (4) was prepared from 2,3-diphenylpropanoyl chloride and aniline. ¹H-NMR (CDCl₃, δ , ppm): 3.10 (2H, *m*, CH₂), 3.60 (1H, *m*, CH), 7.00–7.40 (15H, *m*, 3×ArH); m.p. 168–169 °C.

2,3-Diphenylpropanoyl chloride was synthesized by the reaction of 2,3-diphenylpropanoic acid and thionyl chloride.¹⁰ 2,3-Diphenylpropanoic acid was obtained by the hydrolysis of 2,3-diphenylpropanenitrile, which was obtained by the reaction of phenylacetonitrile and benzyl chloride.¹¹

The other materials were obtained commercially.

The ¹H-NMR spectra were determined on a Varian EM 390 spectrometer (90 MHz) using TMS as the internal standard.

Methods

In a typical procedure for alkylation under microwave irradiation, a mixture of *N*-phenyl--2-phenylacetamide (2.0 mmol), freshly prepared powdered KOH (2.0 mmol), benzyl chloride (2.0 mmol), PTC catalyst (0.20 mmol, if used) was made in a test tube by mixing the substances for 10 s with a spatula. The reaction mixture was then irradiated in a commercial domestic microwave oven (Samsung M182DN). The reaction temperature was measured using an IR thermometer Ebro TN 4088 LC. The reaction was stopped by the addition of water (2.0 ml), and then dichloromethane (5.0 ml) was added. The water layer was separated and washed with dichloromethane (5.0 ml). The organic layers were combined and analyzed by gas chromatography on a DB-1 capillary column (Varian 3400 with Varian integrator 4270), using *n*-hexadecane as an internal standard.

In addition, alkylation was performed in a microwave synthesizer CEM Discover[®] BenchMate (initial power 100 W, set temperature 155 °C, run time 30 s, hold time 0.5–5 min, 25 cm³ flask equipped with a condenser). The subsequent work-up and analysis were same as previously described.

RESULTS AND DISCUSSION

It was shown earlier that when **1** is alkylated under basic conditions different products of alkylation could be obtained.⁵ On the basis of these results, **1** was al-

kylated with benzyl chloride in the presence of powdered potassium hydroxide in a solid–liquid system both with and without a phase-transfer catalyst under microwave irradiation. The alkylation of **1** under basic conditions is given in Scheme 1.

The results of the alkylation of 1 with benzyl chloride at 850 W irradiation power for different reaction times are given in Table I. The yield of the *N*-product increased with increasing reaction time. The highest yield was obtained after 5 min of irradiation. The yields of **3** were between 48.56 and 69.78 %.

TABLE I. Alkylation of **1** with benzyl chloride in the presence of KOH at 850 W microwave irradiation (amount of **1**: 2.0 mmol; amount of KOH: 2.0 mmol; amount of benzyl chloride: 2.0 mmol)

Reaction time min	Yield, %						
Keaction time, min -	1	2	3	4	Other products		
1	50.03	0	48.56	0	1.41		
2	31.23	0	59.94	0.32	8.51		
3	32.80	0	60.58	0.30	6.32		
5	22.93	0	69.78	0	7.29		
7	25.49	0	65.82	0	8.69		
10	25.51	0	63.80	0	10.69		

In addition, the effect of the reactant ratio was investigated. The results of alkylation with excess of benzyl chloride and potassium hydroxide are given in Table II, from which it can be seen that excess benzyl chloride promotes the *N*-alkylation reaction. This was also the case when excess base was used; the maximum yield was achieved with 100 % excess of potassium hydroxide. When excesses of both base and alkylating agent were used, almost all of **1** reacted and the almost highest yield of **2** was achieved. The maximum yield of **3** was about 70 %. In comparison to conventional synthesis (99 % yield of **3**, toluene, reflux, 4 h),⁵ the microwave synthesis gave a lower yield of **3** but no solvent was employed and the reaction time was only 1 min. In addition, reaction was performed in a plain test tube. The highest reaction temperature recorded was 151 °C.

Additional experiments were conducted in a microwave synthesizer CEM Discover[®] BenchMate using an excess of base and alkylating agent, since almost all of **1** reacted in the experiments in a commercial microwave oven. The reaction temperature was set to 155 °C and the initial power was 100 W. In all experiments, the run time was 30 s, while the hold time was varied between 0.5–5 min. The results of alkylation under these conditions are given in Table III. At shorter hold time, the yields of the *O*- and *N*-alkylation products were similar. With increasing hold time, *N*-alkylation prevailed, which is consistent with the fact that the *O*-product is a kinetic product, while the *N*-product is the thermodynamically stable one.² According to the obtained results, *N*-phenyl-2-phenylacetamide is less reactive under microwave irradiation than previously⁸ investigated amides.

TABLE II. Alkylation of **1** in the presence of various quantities of benzyl chloride at 850 W microwave irradiation (amount of **1**: 2.0 mmol; initial amount of base and alkylating agent: 2.0 mmol; reaction time: 1 min)

KOU-PLCU CI	Yield, %						
KOII.FIICII2CI	1	2	3	4	Other products		
1:1	50.03	0	48.56	0	1.41		
1:2	50.85	0.58	36.30	0.57	11.70		
1:3	31.78	1.72	57.60	0	8.90		
2:1	18.54	1.98	71.18	0.74	7.56		
3:1	32.10	0	65.85	0	2.05		
2:2	1.05	12.50	70.83	0.71	14.91		

TABLE III. Alkylation of **1** in a microwave synthesizer CEM Discover[®] BenchMate (amount of **1**: 2.0 mmol; initial amount of base and alkylating agent: 4.0 mmol; initial power: 100 W; set temperature: 155 °C; run time: 30 s)

Hold time min			Yield, %	0	
	1	2	3	4	Other products
0.5	31.33	30.85	34.95	0.61	2.26
1	19.16	8.92	70.20	0.65	1.07
5	8.28	5.57	85.30	0.45	0.40

When 1 was alkylated in the presence of different phase-transfer catalysts under microwave irradiation at 850 W at an equimolar ratio of the reactants, the main product was again found to be the *N*-product, while the *O*-product was a minor product (Table IV). It can also be seen from Table IV that the *C*-product was present in trace amounts only. Although in almost all experiments higher yields were obtained in comparison to the uncatalysed reaction (except when tetrabutylammonium bromide was used), the effects of the phase-transfer catalysts were minor.

Table IV. Alkylation of **1** with benzyl chloride under PTC/OH at 850 W microwave irradiation (amount of **1**: 2.0 mmol; amount of KOH: 2.0 mmol; amount of benzyl chloride: 2.0 mmol; amount of phase-transfer catalyst: 0.20 mmol; reaction time: 1 min)

Quat or catalyst	Counter ion	Yield, %					
Qual of catalyst	Counter Ion	1	2	3	4	Other products	
Et_4N^+	Br-	37.98	0	58.57	0	3.45	
Bu_4N^+	Cl-	41.35	1.08	51.21	0.17	6.19	
	Br-	52.09	0.53	42.27	0	5.11	
	I-	33.35	0.90	59.24	0	6.51	
He ₄ N ⁺	Br-	26.88	10.16	57.00	0	5.96	
TEBA	Cl-	37.51	0.56	53.10	0	8.83	

CONCLUSIONS

When *N*-phenyl-2-phenylacetamide was alkylated with benzyl chloride in the presence of potassium hydroxide under microwave irradiation, the *N*-product was the main product. Excess of base or alkylating agent increased the yield of the *N*-product. Phase-transfer catalysts promote the reaction to a minor extent. The highest yield of the *N*-product of alkylation of *N*-phenyl-2-phenylacetamide in a domestic microwave oven was around 70 % after 1 min at 850 W while in the microwave synthesizer, the yield was around 85 % after 5 min at 155 °C. The *O*-product was obtained in higher yields (up to 30 %) when excesses of base and benzyl chloride were used.

Acknowledgments. The authors acknowledge the financial support of the Ministry of Science of the Republic of Serbia (project 142063). We are thankful to Professor Vera Ćirin-Novta from the University of Novi Sad for her assistance with the experiments in the microwave synthesizer CEM Discover[®] BenchMate.

ИЗВОД

БЕНЗИЛОВАЊЕ *N*-ФЕНИЛ-2-ФЕНИЛАЦЕТАМИДА ПОМОЋУ МИКРОТАЛАСНОГ ЗРАЧЕЊА

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N-Фенил-2-фенилацетамид је алкилован бензил-хлоридом у присуству спрашеног калијум-хидроксида помоћу микроталасног зрачења. Испитиван је утицај односа реактаната и природе међуфазних катализатора на принос очекиваних производа реакције алкиловања. Настајање производа реакције алкиловања (*N*-, *O*- и *C*-производ) је праћено гасном хроматографијом. Утврђено је да је *N*-производ главни производ реакције алкиловања при микроталасном зрачењу. Кад се за алкиловање употребљава вишак бензил-хлорида и калијум-хидроксида у вишем приносу се добија и *O*-производ.

(Примљено 5. јула, ревидирано 21. новембра 2007)

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J. Serb. Chem. Soc. 73 (10) 951–959 (2008) JSCS–3776 JSCS@tmf.bg.ac.yu • www.shd.org.rs/JSCS UDC 632.95+504.53:577.152.1:57.018.8+546.26 Original scientific paper

The impact of atrazine on several biochemical properties of chernozem soil

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(Received 18 January, revised 11 May 2008)

Abstract: The impact of the pesticide atrazine on biochemical processes in soil was investigated. Atrazine loadings of 8.0, 40.0 and 80.0 mg/kg soil were laboratory tested in an experiment set up on a clay loam soil. Dehydrogenase activity, change in biomass carbon, soil respiration and metabolic coefficient were examined. The samples were collected for analysis 1, 7, 14, 21, 30 and 60 days after atrazine application. The acquired data indicated that the effect of atrazine on the biochemical activity of the soil depended on its application rate and duration of activity, and the effect was either stimulating or inhibiting. However, the detected changes were found to be transient, indicating that there is no real risk of the compound disrupting the balance of biochemical processes in soil.

Keywords: atrazine; soil; dehydrogenase; biomass carbon; respiration.

INTRODUCTION

Ever since they were first discovered, pesticides have become an indispensable segment of sound agriculture. Their development and production have soared tremendously and their application rates have increased steadily, so that warning reports of their presence in the environment have become increasingly frequent. Alternatives have therefore been sought since the 1980s and novel and high-safety compounds have been introduced that are not only applied at far lower levels but have more favorable ecotoxicological characteristics as well, so that environmental contamination can, to some extent, be kept under control.^{1,2}

Atrazine, which was first introduced to the market in 1952, belongs to a group of pesticides that are moderately persistent and moderately mobile in soil. The half-life of atrazine varies between several days and several months.³ As the compound has been on the market for a number of years and rather high amounts were applied over wide areas, atrazine residues have been detected both in surface and underground waters.^{4,5} Domestic studies detected 1.0–4.13 μ g L⁻¹ of

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atrazine in surface waters and up to 0.3 μ g L⁻¹ in underground waters.⁶ Its residues in surface and underground waters are consequently causing serious concern for human and animal health and the environment.^{4,5,7}

As a segment of the environment, soil is a complex and dynamic multiphase system with the lithosphere, biosphere, hydrosphere and atmosphere in a perpetual state of balance. Various exogenous factors, primarily heavy metals, pesticides, fertilizers and many others, may disrupt and deteriorate the harmonious state within an ecosystem.⁸ Growing concern for preserving soil as a natural resource have prompted researchers over the past years to focus on studying the changes occurring in soil or those that may occur in the future under certain harmful environmental factors, pesticides being an important one.^{9–11} Various indicators may be used in such research, the most frequently employed being soil enzymes, biomass, respiration, *etc.*

This study is a follow-up to experiments conducted over the past few years in which atrazine residues were examined in Serbian agricultural soils. As atrazine residues were detected in all studied soil samples,⁶ it was assumed that the pesticide and its metabolites may cause certain changes in soil, and in its wider ecosystem, and that experimental data could help assess the risk of disruption of the existing natural balance.

EXPERIMENTAL

The pesticide (herbicide) atrazine (6-chloro- N^2 -ethyl- N^4 -isopropyl-1,3,5-triazine-2,4-diamine) tested in the experiments was a technical grade product of Agan Chemical Manufacturers, Ashdod, Israel. The application loads were: 8.0, 40.0 and 80.0 mg/kg soil. The lowest concentration tested was the recommended application rate (8 mg/kg), and the other two were five and ten times higher doses than that recommended. The experiments were performed using a chernozem soil of a clay loam texture (pH 7.10, organic matter 3.32 %, sand 21 %, silt 49 %, and clay 30 %) at Zemun Polje, Belgrade, Serbia. The soil chosen for the study had never been treated with pesticide before. Various management practices would otherwise have affected the microbial populations of the soil. In this way, it was possible to control the effects of a specific pesticide (atrazine).

Dehydrogenase activity, microbiological biomass carbon and soil respiration were examined as relevant biochemical indicators.¹²⁻¹⁴

Soil samples were collected from the upper layer (0-10 cm), carefully dried, sieved to pass through a 5 mm mesh and stored at 4 °C. Before use, the soils were air-dried at room temperature for 24 h. Each herbicide concentration was pipetted on to the surface of 1 kg of soil before homogenization on a rotating stirrer for 30 min. After homogenization, the soil was portioned into pots. Untreated soil served as the control. The experiments were conducted with four replications. The pots were kept in a controlled-environment chamber at 20 ± 2 °C, 50 % air humidity and a 12–12 h day–night photoperiod throughout the experiment. The soil humidity was maintained at 50 % field capacity. Samples were collected for analysis 1, 7, 14, 21, 30 and 60 days after atrazine application.

The activity of the enzyme dehydrogenase was determined according to Tabatabai.¹⁵ The soil samples were prepared by incubation with triphenyltetrazolium chloride (TTC) under

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moist conditions at 37 °C for 24 h. Triphenylformazan (TPF), which is derived from triphenyltetrazolium chloride (TTC) as a product of enzyme activity, was determined spectophotometrically. Measurements were performed at 485 nm (Gilford stasar III, Model 2400) and the enzyme activity given as μ g TPF/g soil.

Fumigation–extraction¹⁶ was employed to determine microbiological biomass carbon. The samples were fumigated with non-alcohol-containing CHCl₃ under moist conditions for 24 h. After incubation, carbon was extracted with a 0.50 M solution of potassium sulfate and its content determined by titration with a 0.033 M solution of Mohr salt ($(NH_4)_2Fe(SO_4)_2$) in the presence of phenylanthranilic acid as the indicator. Non-fumigated samples were extracted under the same conditions. The microbiological biomass carbon was calculated based on the difference between carbon in the fumigated and non-fumigated samples using a factor 0.38.¹⁷ The results are presented in μ g C/g soil.

The Walter method¹⁸ was employed to determine the soil respiration. The soil samples were incubated with sodium hydroxide under moist conditions at room temperature for 24 h. The carbon dioxide released during soil respiration was absorbed by a 0.10 M solution of sodium hydroxide, and the CO₂ content determined by titration with 0.10 M hydrochloric acid in the presence of an appropriate indicator (phenolphthalein or methyl orange). The results are presented in μ g CO₂/g soil.

The microbiological metabolic coefficient, $q(CO_2)$, was computed from the ratio of the soil respiration intensity and the microbiological biomass.¹⁹

Statistical data processing was performed using PC Anova software. The F-test was applied to all variables and their interactions and, in case of a significant result, the LSD test was applied in individual comparisons. The probability levels 0.05 and 0.01 were used as signify-cance criteria.

RESULTS AND DISCUSSION

Dehydrogenases are soil enzymes catalyzing degradation of organic matter in what is fundamentally a redox process. Soil dehydrogenases are predominantly microbiological in origin and their activities depend on the conditions within the soil ecosystem. Thus a higher enzyme activity indicates a greater intensity of mineralization of the organic matter.^{15,20,21} The results of this experiment show a decreased activity of dehydrogenase for all applied atrazine concentrations from the 1st to the 30th day. The decrease ranged from 12.5-18.2 %, 4.8-24.8 % and 6.6-39.6 % for atrazine loadings of 8.0, 40.0 and 80.0 mg/kg soil (Fig. 1), respectively, and the differences found were statistically significant (P < 0.01). A lower enzyme activity indicates reduced redox intensity in the soil and the reduction degree depended on the concentration and duration of atrazine activity. The enzyme activity was reduced by the harmful activity of atrazine. As pesticides are to some degree environmental toxicants, their noxious activity may be described being manifested mostly through a disturbance of the natural balance. Dehydrogenases, which are free soil enzymes, are very susceptible to unfavorable factors originating from the surrounding environment, including pesticides, *i.e.*, to atrazine in this particular case. There was an increase of enzyme activity from the 30th to the 60th day and the values for treated and untreated soils were RADIVOJEVIĆ et al.

similar at the end of the examination period, except for the highest concentration (80 mg/kg soil), where the value was lower (7.9 %). The obtained experimental data are consistent with the results reported by other authors on the effect of different pesticides on this enzyme.^{22–27}



Data showing the effect of atrazine on the biomass carbon are presented in Fig. 2. The highest biomass carbon (1818 µg C/g soil) was found for an atrazine loading of 8.0 mg/kg soil (7 days after application) and the lowest (986 µg C/g soil) for an atrazine loading of 80.0 mg/kg soil (7 days after application). Reduced biomass carbon under concentrations of 40.0 and 80.0 mg/kg soil was recorded as early as one day after application and the inhibitory effect of the highest concentration (80.0 mg/kg soil) extended 21 days after application. Significant (P < 0.01) increases in the biomass carbon (2.1-45.6 %) were recorded under the lower concentrations of atrazine (8.0 and 40.0 mg/kg soil) from the 7th to the 30th day and at the end of the experiment the increases were 2.1 and 33.3 %, respectively, (Fig. 2). Wardle and Parkinson²⁸ assumed that a most dramatic decrease in the biomass carbon would occur immediately after pesticide application, when the concentration of compound in the soil solution is highest. A number of authors believe²⁹⁻³² that biomass carbon later grows primarily due to the restored populations of live organisms which have adapted to the particular pesticide present in the soil. Hence, a new biomass is formed which is metabolically very active and participates in various biochemical processes in the soil. The biomass carbon is also important for the rate of atrazine degradation in soil. Entry et al.³² monitored the relationship between the degradation rate of atrazine and the microbiological biomass in young and old forests and detected an evident correlation, *i.e.*, that atrazine degraded faster in the older forests with a higher microbiological biomass.

THE EFFECT OF ATRAZINE ON SOIL



Fig 2. Effect of atrazine on microbiological biomass carbon.

There have also been other reports on the activity of different pesticides in relation to biomass carbon. For example, Perucci and Sacrponi^{10,33} found that the effect of rimsulfuron and imazethapyr on biomass carbon depended on the soil moisture. Under reduced moisture, the harmful activity of rimsulfuron lasted 36 h but as long as 72 h under high moisture. Similar findings were also reported by Wardele and Parkinson,²⁸ as well as by Rath,³⁴ in their experiments investigating 2,4-D and glyphosate. Finally, Startton and Stewart³⁵ recorded harmful effects of glyphosate on soil biomass and respiration in Canadian coniferous forests, while 2,4-D has no effect.

The effect of atrazine on soil respiration depended primarily on pesticide concentration (Fig. 3). The respiration intensity ranged between 3.2 (80.0 mg/kg soil, 1 and 7 days after application) and 6.2 μ g CO₂/g soil (40.0 mg/kg soil, 21 days after application). Under the highest concentration of atrazine (80.0 mg/kg soil), the respiration was reduced 13.3–31.8 % from the 1st until the 21st day. Between the 7th and 30th day, an increase in respiration (8.3-56.4 %) was observed under the applied concentrations of 8.0 and 40.0 mg/kg soil. At the end of the experiment (60 days after application), a significant decrease in respiration was detected for all the investigated concentrations. All the found differences were statistically significant (P < 0.01). Literature data show that atrazine, as well as other pesticides, cause a significant inhibition of soil respiration.³³ Some authors $^{36-38}$ found that herbicides from the group of substituted ureas increase soil respiration intensity four weeks after application. The authors believe that increased respiration intensity occurs due to increased activity of microorganisms populating the soil that are able to use the available carbon from the atrazine molecules for their physiological needs. This assumption was later experimentally confirmed by other authors as well.³⁹ Wardle and Parkinson,²⁸ Fleibach and Mader,⁴⁰ and Harden²⁹ found that other pesticides, such as 2,4-D and benomyl, inRADIVOJEVIĆ et al.

creased the intensity of respiration, while glyphosate and dinoseb had no effect on the process. However, other researchers reported that butachlor, alachlor, metolachlor and metribuzin reduced the intensity of this process.^{41–43}



The metabolic coefficient, $q(CO_2)$, is the ratio between soil respiration and biomass carbon,⁴⁴ and a high value indicates an unfavorable environmental factor (*e.g.*, drought, soil cultivation, heavy metals, pesticides, *etc.*).³⁹ In this invest-tigation, increased values of the metabolic coefficient were recorded on the 1st (40.0 mg/kg soil), 7th (40.0 and 80.0 mg/kg soil), 15th (80.0 mg/kg soil), 21st (all examined concentrations) and 30th (40.0 mg/kg soil) day of the experiment. Reduced coefficient values were recorded after the 1st (80.0 mg/kg soil) and 60th day (all employed concentrations). All the detected differences were statistically significant (P < 0.01 and P < 0.05) (Fig. 4).



Fig 4. Metabolic coefficient variation under the effect of atrazine.

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Multiple increases (1.7-, 3- and 5-fold) in the values of the metabolic coefficient have been reported in experiments performed on acid soils,⁴⁵ during drought⁴⁶ or after pesticide treatments. Concerning heavy metals, high coefficient values indicate a change in the energy flow in the basic metabolic processes, and that more carbon is required for their normal continuation.⁴⁶ Wardle et al.²⁸ also found high values of the $q(CO_2)$ coefficient 21 days after glyphosate and dinoseb treatments. Higher levels of this coefficient have been recorded in dinoseb trials, which were to be expected because the pesticide is more toxic than glyphosate. Jones and Ananye³⁹ found that propachlor caused no significant change in the coefficient, while it increased 20-25 % after metalaxyl application, depending on the environmental conditions. Earlier research by these authors showed more harmful effects of metalaxyl in dry than in moist soils, *i.e.*, under conditions less favorable for biosphere development. In the present experiments, increased values of the coefficient $q(CO_2)$ were found from the 1st to the 30th day of the experiment, primarily as a result of the harmful effect of atrazine. The time interval of high $q(CO_2)$ values coincided with the time of significant changes in dehydrogenase, biomass carbon and intensity of respiration. Later (60 days after application), atrazine toxicity ceased, primarily due to its degradation, as well as to the restoration of the populations of living organisms which had adjusted to the new conditions and were able to utilize atrazine molecules as a source of available nutrients and energy for their physiological processes.

CONCLUSIONS

Atrazine was found to cause different effects on the biochemical activity in soil (*i.e.*, dehydrogenase, biomass carbon, respiration and metabolic coefficient). Its influence depended on the application load and duration of activity, and was either stimulating or inhibitory. The impact of atrazine on dehydrogenase activity was consistently negative for each herbicide concentration and depended on the application load. Decreased activity of dehydrogenase under all atrazine concentrations was observed until the 30th day after application. A negative effect was not detected at the end of the incubation period (60th day), when atrazine treated and untreated soils showed similar dehydrogenase values. On the basis of the microbial biomass carbon, soil respiration and metabolic coefficient, non-consistent positive or negative atrazine effects were observed and the effects persisted until the 60th day.

The results suggest difficulties in using biochemical parameters as indicators of the impact of atrazine on soil as different results were acquired depending on the biochemical parameter examined, the application load and post-treatment time. Of the examined parameters, dehydrogenase activity seemed to be the most useful indicator of impact of atrazine on soil environment.

In conclusion, it should be noted that the investigated loadings were either recommended or multiple doses and the observed changes were temporary in char-

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acter and intensity, which suggests that there is no real risk from atrazine of causing a disruption of the existing balance of soil biochemical processes.

ИЗВОД

ДЕЛОВАЊЕ АТРАЗИНА НА НЕКА БИОХЕМИЈСКА СВОЈСТВА ЦРНИЦЕ

Љ. РАДИВОЈЕВИЋ, С. ГАШИЋ, Љ. ШАНТРИЋ и Р. СТАНКОВИЋ-КАЛЕЗИЋ

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У раду је испитивано деловање пестицида (атразина) на биохемијску активност земљишта. Оглед је постављен у лабораторијским условима на земљишту типа глиновита иловача. Атразин је примењен у количинама од 8,0, 40,0 и 80,0 mg/kg земљишта. Праћена је активност ензима дехидрогеназе, биомасе угљеника, респирација (дисање) земљишта као и метаболитички коефицијент. Узорци за анализе узимани су 1, 7, 14, 21, 30 и 60 дана после примене атразина. Добијени резултати су показали да је деловање атразина на биохемијску активност земљишта зависило од примењене количине и дужине деловања, те је у зависности од тога, било стимулативно или инхибиторно. Међутим, утврђене промене су биле пролазног карактера, тако да може да се сматра да нема реалног ризика од нарушавања равнотеже биохемијских процеса у земљишту под утицајем овог једињења.

(Примљено 18. јануара, ревидирано 11. маја 2008)

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J. Serb. Chem. Soc. 73 (10) 961–965 (2008) JSCS–3777 JSCS@tmf.bg.ac.yu • www.shd.org.rs/JSCS UDC *Nepeta rtanjensis:665.52/.54:615.282–188 Short communication

SHORT COMMUNICATION Antifungal activity of *Nepeta rtanjensis* essential oil

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(Received 14 April, revised 27 May 2008)

Abstract: The chemical composition and antifungal activity of the essential oil of an endemic Serbian plant Nepeta rtanjensis Diklić & Milojević was studied. The essential oil was isolated from cultivated plants. Inhibition of mycelia growth of five micromycetes, two Alternaria species originally isolated from N. rtanjensis, Cladosporium cladosporoides, Trichoderma viride and Bipolaris spicifera, were tested using the agar dilution method. The essential oil of N. rtanjensis, the main component of which was $4a\alpha,7\alpha,7\alpha\beta$ -nepetalactone, showed strong antifungal activity against all the tested micromycetes. The minimum inhibitory concentration of N. rtanjensis essential oil ranged from 0.6 to 1.4 µg mL⁻¹. The fungi most sensitive to the tested oil were Alternaria species, while Trichoderma viride was the most resistant.

Keywords: Nepeta rtanjensis; essential oil; antifungal activity.

INTRODUCTION

Nepeta is a genus of perennial or annual herbs from the Lamiaceae family which is found in Asia, central and southern parts of Europe, the Middle East and North Africa.¹ *Nepeta rtanjensis* is an endemic and critically endangered (CR B_{2c}), aromatic plant which grows only on a few localities on the Rtanj Mountain in South-East Serbia.²

Nepeta species are widely used in folk medicine because of their medical properties. The essential oil of *N. rtanjensis* possesses strong antibacterial effect against different strains of *Staphylococcus aureus*, even stronger than most synthetic antibiotics.³

Nepeta species can be divided into two groups: nepetalactone-containing and nepetalactone-free species. The most frequent component in *N. govaniana*, *N.*

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doi: 10.2298/JSC0810961L

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cadmea, N. cephalotes, N. racemosa, N. binaludensis and N. sulforiflora is $4a\alpha,7\alpha,7\alpha\alpha$ -nepetalactone. From the oils of N. nuda spp. albiflora and N. rtanjensis, $4a\alpha,7\alpha,7\alpha\beta$ -nepetalactone was isolated, while N. asterotrichus and N. sintenisii oils are rich in $4a\beta,7\alpha,7\alpha\beta$ -nepetalactone. The main component of the nepetalactone-free species is 1,8-cineol, found in N. heliotropifolia. Caryophyllene oxide was the main component in the oil of N. cilicia, N. betonicifolia and N. nuda. ssp. nuda, while α -pinene was found in the oil of N. glomerulosa and β -caryophyllene in N. fissa oil.⁴

The main component of essential oil of *N. rtanjensis* is $4a\alpha,7\alpha,7a\beta$ -nepetalactone. In wild populations of *N. rtanjensis*, the amount of $4a\alpha,7\alpha,7a\beta$ -nepetalactone in the is oil 86.4 %, while in the oil of cultivated plants, this component is presented with 77.9 %.^{3,4}

It is well known that fungal infection can be a great threat to plant, animal and human health. Medicinal plants are a good source of natural products with strong antimicrobial activities without harmful effects. The use of natural antimicrobial compounds is important in the control of human, animal and plant diseases of microbial origin.

The aim of this investigation was to evaluate the antifungal activity of *N*. *rtanjensis* essential oil against selected fungi.

EXPERIMENTAL

Plant material and isolation of essential oil

Nepeta rtanjensis was collected on experimental fields of the Institute for Biological Research "Siniša Stanković", Belgrade, Serbia. The plants were rapidly micropropagated *in vitro*, transferred to the greenhouse for acclimatization, and subsequently planted in an experimental field.⁵ Herbal material is deposited at the Herbarium of the Institute of Botany and Botanical Garden "Jevremovac", Faculty of Biology, Belgrade (16064 BEOU).

The essential oil was isolated from air-dried aerial parts of *N. rtanjensis*, collected during the pre-flowering stage, by hydrodistillation for 2 h in a Clevenger type apparatus.

Gas chromatography-mass spectrometry (GC/MS)

Analyses of isolated oil were performed by GC/FID and GC/MS on a fused silica capillary column PONA (crosslinked methyl silicone gum, 50 m×0.2 mm, 0.5 μ m film thickness). A Hewlett-Packard, model 5890, series II gas chromatograph equipped with split–splitless injector was used for the GC/FID analysis. Sample solution in ethanol (0.2 %) was injected in split mode (1:100) at 250 °C. The detector temperature was 300 °C (FID), while the column temperature was linearly programmed from 40–280 °C, at a rate of 2 °C min⁻¹. In the case of the GC/MS analysis, a Hewlett-Packard, model 5971A MSD was used. The transfer line was kept at 280 °C. Carrier gas (H₂) flow rate was 1 mL min⁻¹.

Identification of the constituents of the essential oil

Identification of each individual compound was made by comparison of their retention times with those of pure components, matching mass spectral data with those from the Wiley library of 138000 MS spectra. For the library search, a PBM-based software package was used.

Fungal strains used

Two groups of micromycetes were tested: the autochthonous species (*Alternaria* sp. 1 from leaves and *Alternaria* sp. 2 from seeds of *N. rtanjensis*) and selected fungal species (*Cla-dosporium cladosporioides*, *Trichoderma viride* and *Bipolaris spicifera*) from the Mycotheca of the Department of Algology, Mycology and Lichenology, Faculty of Biology, University of Belgrade.

Test for antifungal activity

The fungi were maintained on malt agar (MA). The cultures were stored at + 4 °C and subcultured once in a month. The mycelial growth test with malt agar was used to investigate the antifungal activity of the essential oil.⁶ The minimum inhibitory concentration (*MIC*) of oil necessary for inhibition of mycelial growth of the fungal strain was determined. Different concentrations of essential oil (0.6–1.4 µg mL⁻¹) were diluted in Petri dishes with MA. All fungal species were tested in triplicate. Essential oil was added into MA and poured into Petri dishes. The tested fungi were inoculated at the centre of the plates. The plates were incubated for three weeks at room temperature, the *MIC* was determined after this period. Petri plates with the commercial fungicide, Quadris (0.6–6.0 µg mL⁻¹), were used as controls.

RESULTS AND DISCUSSION

The results of the chemical analysis of the essential oil of *Nepeta rtanjensis* are presented in Table I. The aerial parts of *N. rtanjensis* contained 1.0 % of oil. The main component in this oil was $4a\alpha$, 7α , $7a\beta$ -nepetalactone (79.89 %).

Component	Content, %
<i>a</i> -Pinene	3.3
β-Pinene	0.4
2-Methoxy- <i>p</i> -cresol	1.1
$4a\beta$, 7α , $7a\beta$ -Nepetalactone	6.3
α-Copaene	1.3
$4a\alpha, 7\alpha, 7a\beta$ -Nepetalactone	79.9
Germacrene D	1.8
δ-Cadinene	2.1
Total	96.2

TABLE I. Composition of *N. rtanjensis* essential oil

It was found that the essential oil isolated from *N. rtanjensis* had a strong antifungal activity against all the examined micromycetes. The most efficient impact of *N. rtanjensis* essential oil on mycelia growth *in vitro* was found for the *Alternaria* species (Table II).

TABLE II. Minimal inhibitory concentrations ($MIC / \mu g m L^{-1}$) of *N. rtanjensis* essential oil and Quadris

Micromycetes	Essential oil	Quadris
Alternaria sp. 1	0.8	4.0
Alternaria sp. 2	0.6	4.0
Cladosporium cladosporioides	1.0	3.0
Trichoderma viride	1.4	> 6.0
Bipolaris spicifera	1.0	3.0

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It can be seen that both *Alternaria* species, which were originally isolated from *N. rtanjensis*, showed the highest sensitivity to this oil. Oil concentrations of 0.6–0.8 µg mL⁻¹ inhibited the growth of mycelia of *Alternaria* species. The minimal inhibitory concentration of oil for *Cladosporium cladosporioides* and *Bipolaris spicifera* was 1.0 µg mL⁻¹. The highest *MIC* (1.4 µg mL⁻¹) of oil was against *Trichoderma viride*. The commercial fungicide, Quadris, showed lower antifungal activity than *Nepeta* oil, with *MIC* of 3.0–4.0 µg mL⁻¹. Quadris inhibited mycelial growth of *C. cladosporioides*, *B. spicifera* and *Alternaria* species at 3.0–4.0 µg mL⁻¹. *T. viride* was also the most resistant fungus to Quadris with a *MIC* higher than 6.0 µg mL⁻¹ (Table II).

In previous investigations of the antifungal activity of different oils, it was found that *Alternaria alternata* was more sensitive than *T. viride*.⁷ A strong resistance of *T. viride* was also observed in a previous investigation of the antifungal activity of essential oils. Analyzes of antifungal activity of some essential oils, *Achillea atrata* and Lauraceae plants, showed that *T. viride* was the most resistant fungus.^{8,9}

The present research proved that the essential oil from *N. rtanjensis* has a strong antifungal activity and that this oil can inhibit the growth of the mycelia of some fungi. The results of a previous investigation suggested that *N. rtanjensis* essential oil can inhibit the growth of *Aspergillus niger* colonies.³ According to the literature, this is only data concerning the antifungal activity of *N. rtanjensis* oil. The present research enlarges the number of fungi species known to be sensitive to this oil.

The antifungal activities of essential oils isolated from other *Nepeta* species have been reported. Iridodial β -monoenol acetate isolated from essential oil of *N. leucophyla*, and actidine isolated from *N. clarkei*, showed strong antifungal activity. Iridodial β -monoenol acetate was most effective against *Sclerotium rolfsii*, while actidine was highly active against *Macrophomina phaseolina*. Both fungi are soybean pathogens. The essential oil from *Nepeta hindostana* has an inhibitory effect on *Pythium aphanillermatum*, *P. debaryanum* and *Rhyzoctonia solani*.¹⁰

Due to their low mammalian toxicity, susceptibility to biodegradation and strong antimicrobial activity, essential oils can be used as bioagents.¹¹

Acknowledgment. The presented research was funded by the Ministry of Science of the Republic of Serbia through Project No. 143041.

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извод

АНТИФУНГАЛНА АКТИВНОСТ ЕТАРСКОГ УЉА Nepeta rtanjensis

МИЛИЦА ЉАЉЕВИЋ ГРБИЋ 1 , МИЛОШ СТУПАР 1 , ЈЕЛЕНА ВУКОЈЕВИЋ 1 , МАРИНА СОКОВИЋ 2 , ДАНИЈЕЛА МИШИЋ 2 , ДРАГОЉУБ ГРУБИШИЋ 2 и МИХАИЛО РИСТИЋ 3

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У овом раду презентован је хемијски састав и антифунгална активност етарског уља ендемичне биљке Nepeta rtanjensis Diklić & Milojević. Етарско уље је изоловано из култивисаних биљака. Инхибиција мицелијалног раста пет микромицета, две врсте рода Alternaria, изоловане са N. rtanjensis, Cladosporium cladosporioides, Trichoderma viride и Bipolaris spicifera, тестирана је макродилуционом методом. Етарско уље N. rtanjensis, чија је главна компонента $4a\alpha$,7a, $7a\beta$ -непеталактон показује јаку антифунгалну активност у односу на све тестиране микромицете. Минимална инхибиторна концентрација (MIC) етаског уља била је у распону од 0,6 µg mL⁻¹ до 1,4 µg mL⁻¹. Највећу осетљивост на тестирано уље показале су врсте рода Alternaria док је Trichoderma viride била најотпорнија.

(Примљено 14. априла, ревидирано 27. маја 2008)

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J. Serb. Chem. Soc. 73 (10) 967–978 (2008) JSCS–3778 JSCS@tmf.bg.ac.yu • www.shd.org.rs/JSCS UDC 577.15:547.53:615.281–188 Original scientific paper

The effect of lipophilicity on the antibacterial activity of some 1-benzylbenzimidazole derivatives

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(Received 27 February, revised 13 May 2008)

Abstract: In the present paper, the antibacterial activity of some 1-benzylbenzimidazole derivatives were evaluated against the Gram-negative bacteria *Escherichia coli*. The minimum inhibitory concentration was determined for all the compounds. Quantitative structure–activity relationship (QSAR) was employed to study the effect of the lipophilicity parameters (log P) on the inhibitory activity. Log P values for the target compounds were experimentally determined by the "shake-flask" method and calculated by using eight different software products. Multiple linear regression was used to correlate the log P values and antibacterial activity of the studied benzimidazole derivatives. The results are discussed based on statistical data. The most acceptable QSAR models for the prediction of the antibacterial activity of the investigated series of benzimidazoles were developed. High agreement between the experimental and predicted inhibitory values was obtained. The results of this study indicate that the lipophilicity parameter has a significant effect on the antibacterial activity of this class of compounds, which simplifies the design of new biologically active molecules.

Keywords: benzimidazole derivatives; lipophilicity; quantitative structure–activity relationship; antibacterial; *in vitro* studies.

INTRODUCTION

The benzimidazole nucleus, which is a useful structure for further molecular exploration and for the development of new pharmaceutical compounds, has been studied intensively. The synthesis of benzimidazoles has received a lot of attention owing to the varied biological activity exhibited by a number of these compounds.^{1–7} This class of molecules proved to be very important, as they possess pharmaceutical properties, including antibacterial, against different strains of Gram-positive and Gram-negative bacteria,^{8–10} antifungal³ and herbicidal¹¹ activity. It is also well-known that these molecules are present in a variety of anal-

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doi: 10.2298/JSC0810967P

gesic,¹² anti-oxidant,^{13,14} anti-allergic^{15,16} and antitumoral¹⁷ agents. Many derivatives of benzimidazole show antiparasitic¹⁸ and anthelmintic¹⁹ activities. In addition, they were confirmed to have moderate *in vitro* anti-HIV activity.^{20,21}

The success with these groups of molecules stimulated the search for new biologically active derivatives. Understanding the role of chemical structure on influencing biological activity is very important.^{22,23} Progress in the use of quantitative structure–activity relationship (QSAR) methods has shown the importance of the hydrophobic or lipophilic nature of biologically active molecules. The lipophilicity modifies the penetration of bioactive molecules through the apolar cell membranes. This property is usually characterized by the partition coefficient (log *P*), which is essentially determined from distribution studies of the compound between an immiscible polar and non-polar solvent pair. This quantitative descriptor of lipophilicity is one of the key determinants of pharmaco-kinetic properties.^{24–26} Knowing the exact values for this parameter, it is possible to predict the inhibitory activity of the drugs.

In this context, the aim of the present study was to investigate the activity of different substituted benzimidazoles against the Gram-negative bacteria *Escherichia coli* and to study the quantitative effect of lipophilicity on antibacterial activity. The objective of this study was to develop a rapid and reliable method for predicting the antibacterial activity of this class of molecules, as well as to determine the best log *P* values affording the most significant multilinear QSAR models, which link the structure of these compounds with their inhibitory activity.

EXPERIMENTAL

Modeling of compounds and calculation of lipophilicity parameters

The investigated compounds (Table I) were synthesized by a procedure described earlier.²⁷ The free on-line JME molecular editor software was used to model these molecules in SMILES (simplified molecular input line entry system) format. The SMILES notation created by the structure drawing program CambridgeSoft's ChemDrawPro was used as chemical structure input for all programs, except HyperChem 7.5 (HyperCube Inc., Version 7.5).²⁸

TABLE I. Structural formula of the compounds

		CH ₂	$CH_2 \longrightarrow$			
Compound	R ₁	R ₂	R ₃	R_4		
I-CH ₃	CH ₃	Н	CH ₃	CH ₃		
I-Cl	Cl	Н	CH_3	CH ₃		
I-F	F	Н	CH_3	CH ₃		
I-OCH ₃	OCH ₃	Н	CH_3	CH ₃		
II-CH ₃	CH_3	$\rm NH_2$	Н	Н		
II-Cl	Cl	NH_2	Н	Н		
II-F	F	$\rm NH_2$	Н	Н		
II-OCH ₃	OCH ₃	NH ₂	Н	Н		



TABLE I. Continu	ed			
Compound	R ₁	R ₂	R ₃	R ₄
III-CH ₃	CH ₃	NH ₂	CH ₃	CH ₃
III-Cl	Cl	NH_2	CH ₃	CH ₃
III-F	F	NH_2	CH ₃	CH ₃
III-OCH ₃	OCH ₃	$\rm NH_2$	CH_3	CH_3

The lipophilicity parameters, based on log P, for all the compounds were experimentally determined (the "shake-flask" method) and their values calculated using different theoretical procedures from internet data (log P_{Hyper} , CSlog P, milog P, ALOGP, IAlogP, CLOGP, log Kow and XLOGP) (Table II).

TABLE II. Lipophilicity descriptors experimentally determined by "shake-flask" method and those calculated using different software

	Log P								
Cmpd.	Experi-	- Calculated							
	mental	$\operatorname{Log} P_{\operatorname{Hyper}}$	CSlog P	milog P	ALOGP	IAlogP	CLOGP	log Kow	XLOGP
I-CH ₃	4.85	3.48	4.66	4.32	3.96	4.00	4.80	5.13	4.64
I-Cl	5.05	3.75	4.46	4.55	4.27	4.27	5.01	5.23	4.83
I-F	4.50	2.70	3.85	4.03	3.65	3.34	4.44	4.78	3.85
I-OCH ₃	4.28	2.31	4.29	3.93	3.55	3.75	4.22	4.67	4.29
II-CH ₃	3.60	2.75	3.06	3.32	3.14	2.70	3.56	3.68	3.44
II-Cl	3.85	2.80	3.48	3.55	3.60	3.06	3.78	3.78	3.62
II-F	3.25	2.42	2.18	3.03	3.08	3.16	3.21	3.33	3.17
II-OCH ₃	3.04	2.03	2.85	2.93	2.83	2.57	2.98	3.21	2.92
III-CH ₃	4.55	3.06	4.13	4.12	3.85	3.36	4.51	4.77	4.32
III-Cl	4.78	3.01	4.41	4.35	4.18	3.65	4.72	4.87	4.50
III-F	4.20	2.72	3.28	3.84	3.55	2.94	4.16	4.43	4.04
III-OCH ₃	3.97	2.34	3.90	3.73	3.46	3.20	3.93	4.31	3.80

"Shake-flask" method

Partition coefficients (*P*) for benzimidazoles between *n*-octanol and phosphate buffer were determined at 25 °C. Before the partitioning of the benzimidazoles, the buffer (0.15 mol L⁻¹, pH 7.4) and *n*-octanol (99 %, Sigma, USA) were saturated with each other. The benzimidazoles were dissolved in ethanol (96 %, Zorka, Serbia) at a concentration of 2 mg mL⁻¹ to give the stock solutions. Calibration was performed in exactly the same manner as the partitioning, except that *n*-octanol was not used. The amounts of the sample were chosen so that the absorbance ($\lambda = 252$ nm) was between 0.10 and 0.80. The partitioning experiments were performed in the systems *n*-octanol/phosphate buffer 1:20, 1:30, 1:70 and 1:80 (v/v). All the solutions were pipetted into glass vials; the *n*-octanol and stock solution were added with a microliter syringe. The phases were shaken together on a mechanical shaker (Viggo, Sweden) for 30 min, centrifuged (Rotofix, Switzerland) at 2500 rpm for 20 min to afford complete phase separation, and the *n*-octanol phase was removed. The absorbance of the buffer phase was measured using a Shimadzu UV/Vis spectrophotometer (Japan) at 252 nm. Log *P* values were calculated using Eq. (1):

$$\log P = \log\left(\frac{y-x}{x}\frac{V_{\text{buffer}}}{V_{n-\text{octanol}}}\right)$$
(1)

where *P* is partition coefficient, *y* is total mass of benzimidazole derivative (mg), *x* is the mass of benzimidazole derivative in the buffer phase after partitioning (mg), V_{buffer} is the volume of phosphate buffer (mL) and $V_{n-\text{octanol}}$ is the volume of *n*-octanol (mL). Each experimental log *P* value is the average of five determinations.

Calculation methods

A number of different computer programs for the calculation (prediction) of the lipophilicity of chemical compounds, based on their structure, have recently been developed.

Log P_{Hyper} . The computer program HyperChem 7.5 predicts log *P* values using the atom-additive method according to Ghose, Prichett and Crippen.²⁹ The program lists atom contributions for each atom type and calculates the log *P* value summing up all the atom contributions.

CSlog P. This program is based on topological structure descriptors and electropological state (E-state) indices.³⁰

milog P. The milog P 1.2 program calculates log P values as the sum of group contributions and correction factors.³¹

ALOGP. The ALOGPS 2.1 package includes programs to predict lipophilicity and aqueous solubility of chemical compounds. The method is based on atom-type E-state indices and the associative neural network modeling was developed by Tetko *et al.*³² This method combines electronic and topological characters to predict lipophilicity of the analyzed molecules.

IAlogP. This is another calculation program which predicts lipophilicity of chemical compounds using neural network algorithms and Molconn-Z indices, including E-state indices for atom types.³³

CLOGP. The CLOGP 4.0 program is based on the fragmental method developed by Leo and Hansch³⁴ and has become the standard in the field of rational drug design.

LogKOW. The KoWWin program calculates log *P* values of organic compounds using the atom/fragment contribution (AFC) method developed by Syracuse Research Corporation (SRC).³⁵

XLOGP. The XLOGP 2.0 is a computer program based on additive atomic contributions and calculates log P values according to Wang, Fu and Lai.^{36,37}

The complete regression analysis, including linear, non-linear and multi-linear regression (MLR), were carried out by PASS 2005, GESS 2006, NCSS Statistical Software.³⁸

Antibacterial investigations

All the 1-benzylbenzimidazole derivatives were evaluated for their *in vitr*o growth inhibitory activity against Gram-negative bacteria *Escherichia coli* (ATCC 25922). Antibacterial activities of the compounds were tested by the disc-diffusion method under standard conditions using Mueller-Hinton agar medium as described by NCCLS.³⁹ The investigated isolate of bacteria was seeded in tubes with nutrient broth (NB). The seeded NB (1 cm³) was homogenized in tubes with 9 cm³ of melted (45 °C) nutrient agar (NA). The homogenous suspension was poured into Petri dishes. Discs of filter paper (diameter 5 mm) were placed on the cool medium. After cooling on the formed solid medium, 2×10^{-5} dm³ of the investigated compounds were added by micropipette. After incubation for 24 h at 25–27 °C, the diameters of the inhibition (sterile) zone (including disc) were measured (in mm). A diameter of the inhibition zone greater than 8 mm indicates the tested compound was active against the microorganism. Every test was performed in triplicate.

Minimum inhibitory concentration (*MIC*) was determined by the agar dilution method according to guidelines established by the NCCLS standard M7-A5.⁴⁰ The *MIC* value of tested benzimidazoles is defined as the lowest concentration of the compound at which no growth of the strain was observed in a period of time and under specified experimental conditions. Stock solutions of the compounds were prepared in dimethylformamide (DMF). Further dilutions were performed with distilled water. The concentration range of the compounds tested
was between 6.25–50 µg ml⁻¹. The inoculated plates were then incubated at 35 °C for 16–20 h. A control using DMF without any test compound was included. There was no inhibitory activity in the wells containing only DMF. The *MIC* values of the benzimidazoles tested were obtained as µg ml⁻¹. For further QSAR analyses, the negative logarithms of molar *MIC*s (log $(1/c_{MIC})$) were used. In order to classify the antibacterial activity comparisons were established with antibacterial agents currently employed in therapeutic treatment. The *MIC*s were compared with those of ampicillin and gentamicin, which were screened under similar conditions as the tested compounds.

RESULTS AND DISCUSSION

The values of antibacterial activity of the benzimidazole derivatives against the tested Gram-negative bacteria are summarized in Table III. The screening results revealed that the investigated compounds expressed inhibitory activity against *Escherichia coli*. Compounds with a high log $1/c_{MIC}$ (or low *MIC*) are the best antibacterials.

TABLE III. Summary of the antibacterial screening

Compound	$MIC / \mu g ml^{-1}$	$\log(1/c_{MIC})$
I-CH ₃	6.25	4.602
I-Cl	6.25	4.637
I-F	6.25	4.609
I-OCH ₃	12.5	4.328
II-CH ₃	12.5	4.278
II-Cl	12.5	4.314
II-F	25.0	3.981
II-OCH ₃	50.0	3.704
III-CH ₃	6.25	4.627
III-Cl	6.25	4.659
III-F	12.5	4.333
III-OCH ₃	12.5	4.352
Ampicillin	12.5	4.446
Gentamicin	0.780	5.787

In order to identify the effect of lipophilicity on the inhibitory activity, QSAR studies of title compounds were performed. A set of benzimidazoles consisting of 12 molecules was used for the generation of a multilinear regression model. The reference drugs were not included in the generation of the model as they belong to a different structural series. An attempt was made to find the structural requirement for the inhibition of Gram-negative *E. coli* using the QSAR Hansch approach on benzimidazole derivatives. To obtain the quantitative effects of the structural parameters of benzimidazole derivatives on their antibacterial activity, QSAR analysis with nine different partition coefficients (log *P*) was operated. First, the correlation of each one of the log *P* values with each other was calculated. The resulting correlation matrix is represented in Table IV. As is indicated, the calculated partition coefficients were in good correlation with each other, especially milog P, CLOGP, XLOGP and log Kow, as well as the experimentally obtained log *P* value.

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TABLE IV. Correlation (r) matrix for the lipophilicity descriptors used in this study

Procedure	"shake- flask"	$\log P_{\text{Hyper}}$	CSlog P	milog P	ALOGP	IAlog P	CLOGP	log Kow	XLOGP
"shake-flask"	1	0.8019	0.9030	0.9971	0.9619	0.8439	0.9998	0.9842	0.9689
$\log P_{Hyper}$		1	0.6199	0.7800	0.8337	0.7190	0.8052	0.7194	0.7913
CSlog P			1	0.9176	0.8550	0.7921	0.8997	0.9170	0.8906
milog P				1	0.9654	0.8434	0.9969	0.9844	0.9928
ALOGP					1	0.8390	0.9609	0.9092	0.9469
IAlog P						1	0.8437	0.8419	0.8513
CLOGP							1	0.9847	0.9979
log Kow								1	0.9894
XLOGP									1

Usually, lipophilicity parameters are linearly related to pharmacological activity (*MICs*), but in the more general case, this relationship is not linear.^{41,42} Therefore, a complete regression analysis was made including linear, quadratic and cubic relationships. It is apparent from the data presented in Table V that the fitting equations improved when resorting to higher order (second or third order) polynomials.

TABLE V. Correlation coefficients (r) calculated for the relationship between log $1/c_{MIC}$ and different log P values

Drogoduro	$Log (1/c_{MIC}) = a log P + b$	$Log (1/c_{MIC}) = a log P^2 +$	$Log (1/c_{MIC}) = a log P^3 +$
rioceduie		$+ b\log P + c$	$+ b\log P^2 + c\log P + d$
"shake-flask"	0.9384	0.9626	0.9664
$\log P_{\rm Hyper}$	0.7628	0.8450	0.8490
CSlog P	0.8206	0.8211	0.8478
milog P	0.9282	0.9500	0.9549
ALOGP	0.9077	0.9486	0.9493
IAlog P	0.7032	0.7646	0.7757
CLOGP	0.9389	0.9640	0.9672
log Kow	0.9053	0.9246	0.9443
XLOGP	0.8706	0.9226	0.9359

Data from Table V indicates that only two of the aforementioned $\log P$ values are highly correlated with the measured activity. However, the introduction of a second parameter improved the statistical indices of the QSAR models but the best QSAR models were obtained with three variables with second order polynomials. The resulting models are as follows:

$$\begin{split} & \text{Log} \left(1/c_{MIC} \right) = 0.140 \text{CLOGP}^2 + 0.103 \text{log Kow}^2 - 0.491 \text{XLOGP}^2 - \\ & - 0.302 \text{Clog } P - 1.191 \text{log Kow} + 3.756 \text{XLOGP} - 0.632 \\ & n = 12; \ r = 0.9855; \ s = 0.0744; \ F = 28 \end{split} \tag{2}$$

$$-5.246 \text{ALOGP} - 6.145 \log \text{Kow} + 11.544 \text{XLOGP} + 3.277$$
(4)

$$n = 12; r = 0.9843; s = 0.0775; F = 25.326$$

The statistical quality of the resulting models, as depicted in Eqs. (2)–(4), is given by the correlation coefficient, r, the standard error of estimation, s, and the probability factor related to the *F*-ratio, *F*. It is noteworthy that all these equations were derived using the entire data set of compounds (n = 12) and no outliers were identified. The *F*-values obtained in Eqs. (2)–(4) are statistically significant at the 99 % level, since all the calculated *F* values are higher than the tabulated values.

To estimate the quality with regards to predictive ability of this model, the cross-validation statistical technique was applied. This is the most common validation technique, where a number of modified data sets are created by deleting, in each case, one or smaller group of objects from the data in such a way that each object is taken away once and only once. For each reduced data set, the model is calculated, and responses for the deleted objects are predicted from the model. The simplest and most general cross-validation procedure is the leave-one-out technique (LOO technique). The estimation of the models quality was based on cross-validated parameters *viz.*, the predicted residual sum of squares, *PRESS*, the total sum of squares deviation, *SSY*, the uncertainty of prediction, *S_{PRESS}*, the cross-validated correlation coefficient, r_{CV}^2 , and the adjusted correlation coefficient, r_{adi}^2 (Table VI).

TABLE VI. C	Cross-validation	parameters
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Equation	PRESS	SSY	PRESS/SSY	S _{PRESS}	$r_{\rm CV}^2$	$r_{\rm adj}^2$
(2)	0.1633	0.9417	0.1734	0.1166	0.8266	0.9366
(3)	0.2114	0.9417	0.2245	0.1327	0.7755	0.9285
(4)	0.2037	0.9417	0.2163	0.1303	0.7837	0.9299

PRESS is an important cross-validation parameter as it is a good approximation of the real predictive error of the models. Its value being less than the *SSY* indicates whether a model predicts better than chance and whether it can be considered statistically significant. Thus, in view of this, all the three proposed models are statistically significant. Furthermore, to be a reasonable QSAR model, the *PRESS/SSY* ratio should be less than 0.40. The data presented in Table VI indicate that this ratio is < 0.23 for all the developed models. From the *PRESS* and *SSY*, the r_{CV}^2 and *S*_{PRESS} statistics can be easily calculated:

$$r_{\rm CV}^2 = 1 - PRESS/SSY \tag{5}$$

$$S_{PRESS} = \sqrt{\frac{PRESS}{n}} \tag{6}$$

The high r_{CV}^2 values observed for all the proposed QSAR models are indicative of their reliability in the prediction of inhibitory activity. However, the only way to estimate the true predictive power of a model is to test its ability to predict accurately the biological activities of compounds. In order to verify the predictive power of the developed models, the predicted log (1/*c_{MIC}*) values of the

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investigated benzimidazoles were calculated using Eqs. (2)–(4) and compared with the experimental values. Based on the magnitude of the residue, there is a close agreement between the observed and calculated inhibitory activities (Table VII). Furthermore, plots of the linear regression predicted log $1/c_{MIC}$ values against the observed log $(1/c_{MIC})$ values also favor the models expressed by Eqs. (2)–(4) (Fig. 1).

TABLE VII. Predicted log $(1/c_{MIC})$	values of the benzimi	dazoles tested against <i>E. coli</i>
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Compound		Pre	dicting equat	ions and residu	ues	
Compound -	Eq. (2)	Residue	Eq. (3)	Residue	Eq. (4)	Residue
I-CH ₃	4.595	0.007	4.590	0.012	4.573	0.029
I-Cl	4.637	0.000	4.638	-0.001	4.628	0.009
I-F	4.625	-0.016	4.618	-0.009	4.616	-0.007
I-OCH ₃	4.343	-0.015	4.389	-0.061	4.388	-0.060
II-CH ₃	4.185	0.093	4.212	0.066	4.285	-0.007
II-Cl	4.334	-0.020	4.284	0.030	4.300	0.014
II-F	3.986	-0.005	4.004	-0.023	4.017	-0.036
II-OCH ₃	3.727	-0.023	3.717	-0.013	3.709	-0.005
III-CH ₃	4.573	0.054	4.648	-0.021	4.557	0.070
III-Cl	4.657	0.002	4.651	0.008	4.712	-0.053
III-F	4.435	-0.102	4.327	0.006	4.336	-0.003
III-OCH ₃	4.342	0.010	4.342	0.010	4.347	0.005





Fig. 1. Plots of the predicted *versus* the experimentally observed inhibitory activity of theinvestigated 1-benzylbenz-imidazoles against *E. coli*.

In order to investigate the existence of a systemic error in the development of the QSAR models, the residuals of the predicted log $(1/c_{MIC})$ values were plotted against the observed log $(1/c_{MIC})$ values (Fig. 2). The propagation of the residuals on both sides of zero indicates that no systemic error exists in the development of regression models, as suggested by Jalali-Heravi and Kyani.⁴³



From the three above presented models, it can be concluded that a strong influence of the partition coefficient, $\log P$, is important for antibacterial activity and this parameter is usually related to the pharmacological activity.^{41,44} This evidence was clearly described in the lipid theory advanced by Meyer and Overton. According to this theory, $\log P$ is a measure of hydrophobicity, which is important not only for the penetration and distribution of a drug, but also for the interaction of the drug with receptors. Therefore, it can be suggested that lipophilic properties should be checked in the design of potent antibacterial agents as they are deciding factors for their activity.

CONCLUSIONS

From the results discussed above, it can be concluded that the investigated 1-benzylbenzimidazole derivatives showed *in vitro* inhibitory activity against the

Gram-negative bacteria Escherichia coli. QSAR analyses were employed to study the quantitative effects of the lipophilicity of the benzimidazoles on their antibacterial activity. Different lipophilicity parameters were experimentally determined by the "shake-flask" method and calculated using eight different software products. A complete regression analysis was performed in which linear, quadratic and cubic relationships between the $\log P$ values and the antibacterial activity $(\log (1/c_{MIC}))$ were employed. The fitting equations improved when higher order (second or third order) polynomials were used. Three high quality non-linear structure-activity models were derived between the log $(1/c_{MIC})$ and three different log P values. The obtained mathematical models were used to predict the inhibittory activity of the investigated benzimidazoles and close agreement between the experimental and predicted values was found. The low residual activity and high cross-validated r^2 values (r_{CV}^2) observed indicate the predictive ability of the developed QSAR models. It indicates that these models can be successfully applied to predict the antibacterial activity of this class of molecules. It can be concluded that the partition coefficient, log P, has a strong influence on the antibacterial activity and this parameter is usually related to pharmacological activity.

Acknowledgement. These results are the part of the project No. 142028, supported by the Ministry of Science of the Republic of Serbia.

ИЗВОД

УТИЦАЈ ЛИПОФИЛНОСТИ НА АНТИБАКТЕРИЈСКУ АКТИВНОСТ НЕКИХ ДЕРИВАТА БЕНЗИМИДАЗОЛА

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У овом раду испитана је антибактеријска активност неких деривата 1-бензилбензимидазола на грам-негативну бактерију *Escherichia coli*. Испитивани бензимидазоли *in vitro* показују антибактеријску активност и за сва једињења је одређена минимална инхибиторна концентрација. Применом QSAR (quantitative structure–activity relationship) анализе испитане су зависности између инхибиторне активности и параметара липофилности, log P. За испитивана једињења, log P вредности су одређене експерименталном "shake-flask" методом и израчунате су помођу осам различитих рачунарских програма. Методом вишеструке регресије испитане су корелације између log P вредности и антибактеријске активности деривата бензимидазола. Резултати су продискутовани на основу статистичких података. Развијени су најприхватљивији математички модели за предвиђање антибактеријске активности у оквиру испитиване серије бензимидазола. Добијено је веома добро слагање између експериментално одређених и предвиђених вредности има значајан утицај на антибактеријску активност испитиване класе једињења, што олакшава дизајнирање нових биолошки активних молекула.

(Примљено 27. фебруара, ревидирано 13. маја 2008)

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J. Serb. Chem. Soc. 73 (10) 979–988 (2008) JSCS–3779 JSCS@tmf.bg.ac.yu • www.shd.org.rs/JSCS UDC 546.34+537.622.4:542.913:547.476.1 Original scientific paper

Synthesis of lithium ferrites from polymetallic carboxylates

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(Received 6 March, revised 20 May 2008)

Abstract: Lithium ferrite was prepared by the thermal decomposition of three polynuclear complex compounds containing as ligands the anions of malic, tartaric and gluconic acid: $(NH_4)_2[Fe_{2.5}Li_{0.5}(C_4H_4O_5)_3(OH)_4(H_2O)_2]\cdot 4H_2O$ (I), $(NH_4)_6[Fe_{2.5}Li_{0.5}(C_4H_4O_6)_3(OH)_8]\cdot 2H_2O$ (II) and $(NH_4)_2[Fe_{2.5}Li_{0.5}(C_6H_{11}O_7)_3(OH)_7]$ (III). The polynuclear complex precursors were characterized by chemical analysis, IR and UV–Vis spectra, magnetic measurements and thermal analysis. The obtained lithium ferrites were characterized by XRD, scanning electron microscopy, IR spectra and magnetic measurements. The single α -Li_{0.5}Fe_{2.5}O₄ phase was obtained by thermal decomposition of the tartarate complex annealed at 700 °C for 1 h. The magnetization value ≈ 50 emu g⁻¹ is lower than that obtained for the bulk lithium ferrite due to the nanostructural character of the ferrite. The particle size was smaller than 100 nm.

Keywords: lithium ferrite; chemical synthesis; thermogravimetric analysis (TGA); X-ray diffraction; magnetic properties.

INTRODUCTION

Nanocrystalline lithium ferrite has been investigated in the last years due to its potential use in the microwave field as a replacement for garnets or as a memory core.^{1,2} Lithium ferrite has important magnetic properties, *i.e.*, a high Néel temperature, a rectangular hysteresis loop and a high dielectric constant.^{3,4}

Lithium ferrite is an inverse spinel which exists in two crystalline forms: ordered, α -Li_{0.5}Fe_{2.5}O₄, and disordered, β -Li_{0.5}Fe_{2.5}O₄.⁵⁻¹⁰

The method of preparation plays a very important role in determining the chemical, structural and magnetic properties of spinelic ferrites. The conventional ceramic method, which involves high-temperatures sintering, often result in a low quality material; the volatility of lithium above 1000 °C affects the magnetic properties and the resistivity.⁶ In order to overcome such difficulties, a

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number of wet chemical methods have been developed to prepare LiFe₅O₈ at low temperatures. They include the sol–gel method,^{11,12} the microemulsion method,¹³ hydrothermal transformations of hydroxide precursors,¹⁴ freeze-drying of Li, Fe-formates,¹⁵ the citrate precursors method,^{10,16} and autocombustion.¹⁷

The nature of the precursors is very important in the synthesis of spinelic ferrites. Polynuclear coordination compounds are preferred as precursors because they can lead directly to mixed oxides by thermal decomposition.¹⁸ A new synthesis route based on the thermal decomposition of compounds with acetylacetone-(2,4-pentadione) ligands was applied to obtain nano-sized Li-ferrites.¹⁹

The synthesis of nanoferrites by thermal decomposition of polynuclear complex compounds is a non-conventional method which belongs to "chimie douce" (soft chemistry). This method is sometimes referred to as the complexation method for the synthesis of ferrites. The polynuclear coordination compounds, which may be used as precursors for ferrites, should generate only volatile products on decomposition. Ligands that largely satisfy this requirement are the anions of carboxylic and hydroxycarboxylic acids, *i.e.*, acetate, oxalate, citrate, *etc*.

The goals of the present study were to synthesize polynuclear coordination compounds, precursors of lithium ferrite, containing as ligands the anions of malic, tartaric and gluconic acid; to characterize these complex compounds by IR and UV–Vis spectroscopy and magnetic measurements; to decompose them at low temperatures in order to obtain lithium ferrite; and to characterize the samples of lithium ferrite by IR spectroscopy, XRD analysis, SEM and magnetic measurements.

EXPERIMENTAL

All chemicals: $Fe(NO_3)_3.9H_2O$, LiNO₃, malic acid, tartaric acid and δ -gluconolactone were of reagent quality (Merck). The precursors – polynuclear coordination compounds were prepared as follows.

Iron and lithium nitrates were dissolved in the minimum amount of water and mixed with an aqueous solution of carboxylic acid in a 2.5:0.5:4 ratio $Fe^{3+}:Li^+:malic$ (tartaric) acid and 2.5:0.5:8 ratio $Fe^{3+}:Li^+:\mathcal{E}$ -gluconolactone.

Ethanol was added to the final solution until a yellow precipitate formed. The pH was raised to 5.5–6.0 by adding NH₃:H₂O (25 %):ethanol solution (1:1). The yellow polynuclear compounds were filtered, washed with ethanol and dried over P_4O_{10} .

The metal content of the polynuclear compounds was determined by atomic absorption spectroscopy with an SAA1 instrument and by gravimetric techniques; the C, H, and N values were obtained using a Carbo Erba Model 1108 CHNS-O elemental analyzer.

The IR spectra (KBr pellets) of the polynuclear coordination compounds and the lithium ferrites were recorded on a Bio-Rad FTS-135 spectrophotometer in the 4000–400 cm⁻¹ region.

The thermogravimetric, TG, differential TG, DTG, and differential thermal analysis, DTA, curves were simultaneously recorded under an air atmosphere using a Shimadzu DTG-TA--51H instrument. The reference material was Al_2O_3 . The heating rate was 10 °C min⁻¹ in the temperature range 20–800 °C.

The XRD patterns were recorded on a Rigaku-Multiflex X-ray diffractometer using CuK_{α} radiation. For quantitative analysis, the step scanning technique was applied in the 2θ

range 20–80° with a scan speed of 2° min⁻¹. The particles size was determined using the Scherrer Equation.

The morphological analysis of the samples was performed by scanning electron microscopy (SEM) using a Hitachi S2600N electron microscope (image analysis with a secondary electron detector (SE)).

The magnetic measurements on the complex compounds and lithium ferrite samples are performed with a Faraday balance (HgCo(SCN)₄, $\chi_g = 16.44 \times 10^{-6}$ cgs units and metallic Ni as calibrants) and a magnetometer based on the extraction method with a resolution of 10^{-4} emu, accessible 1.5–300 K, magnetic field maximum 11 T, increment 10 Oe.

RESULTS AND DISCUSSION

Characterization of the polynuclear complex compounds

Elemental analyses of the polynuclear coordination compounds containing as ligands: malate, tartarate and gluconate anions (I, II, and III, respectively), were consistent with the formula:

 $(NH_4)_2[Fe_{2.5}Li_{0.5}(C_4H_4O_5)_3(OH)_4(H_2O)_2] \cdot 4H_2O(I)$. Anal. Calcd.: Fe, 18.63; Li, 0.465; C, 19.16; H, 4.79; N, 3.73 %. Found: Fe, 18.76; Li, 0.46; C, 19.28; H, 4.88; N, 3.84 %.

 $(NH_4)_6[Fe_{2.5}Li_{0.5}(C_4H_4O_6)_3(OH)_8] \cdot 2H_2O$ (**II**). Anal. Calcd.: Fe, 16.13; Li, 0.403; C, 16.60; H, 5.53; N, 9.68 %. Found: Fe, 15.92; Li, 0.395; C, 16.64; H, 5.60; N, 9.85 %.

 $(NH_4)_2[Fe_{2.5}Li_{0.5}(C_6H_{11}O_7)_3(OH)_7]$ (III). Anal Calcd.: Fe, 15.84; Li, 0.396; C, 24.44; H, 5.43; N, 3.17 %.Found: Fe, 16.03; Li, 0.40; C, 24.46; H, 5.26; N, 3.26 %.

The IR spectra of these polynuclear complex compounds suggest that the hydroxycarboxylate anions are coordinated to the metal ions through the COO^- and C–OH groups.

The band at 1700–1730 cm⁻¹ of the free carboxylic acid, assigned to $v_{C=O}$, is replaced in the spectra of the compounds by two intense bands, $v_{as}(OCO) \approx 1620-1630$ cm⁻¹ and $v_s(OCO) \approx 1384$ cm⁻¹. On the basis of spectroscopic criteria,²⁰ the magnitude of the separation $\Delta v = v_{as} - v_s$ may be indicative for establishing the mode of coordination of carboxylate ions. Thus, Δv values in the range 140–160 cm⁻¹, *i.e.*, higher than those observed for ionic compounds ($\Delta v(Na_2L) = 120$ cm⁻¹) suggest a bridging bidentate bonding. On the other hand, values of $\Delta v > 180$ cm⁻¹ are characteristic for monodentate coordination.

The splitting of the $v_{as}(OCO)$ vibration detected in the IR spectrum of the malate complex, compound I, suggests two different coordination modes for the $C_4H_4O_5^{2-}$: monodentate bonding ($v_{as}(OCO) \approx 1620 \text{ cm}^{-1}$; $v_s(OCO) \approx 1380 \text{ cm}^{-1}$) and bridging bidentate bonding ($v_{as}(OCO) \approx 1560 \text{ cm}^{-1}$; $v_s(OCO) \approx 1380 \text{ cm}^{-1}$).²⁰

For compounds II ($v_{as}(OCO) \approx 1621 \text{ cm}^{-1}$; $v_s(OCO) \approx 1385 \text{ cm}^{-1}$) and III ($v_{as}(OCO) \approx 1630 \text{ cm}^{-1}$; $v_s(OCO) \approx 1384 \text{ cm}^{-1}$), only the monodentate coordination mode was detected.

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An analysis of these spectra within the 1000–1100 cm⁻¹ range reveals a considerable difference between the spectra of the free acids and the spectra of the compounds. The peak assigned to the C–O stretching vibration of the secondary OH groups, which appears at $\approx 1100-1097$ cm⁻¹ in the spectra of the free acids, is splitted and shifted towards lower frequencies (1080–1040 cm⁻¹) in the IR spectra of the coordination compounds. The splitting can be explained by a different bonding of the secondary OH groups present in the molecule of the hydroxycarboxylate anions. One can advance the hypothesis that the secondary OH groups coordinate to two different metal ions.

The reflectance spectra of the polynuclear complex compounds revealed the presence of weak forbidden transition bands with the Fe³⁺ (d⁵) in an octahedral high spin configuration (${}^{6}A_{1g} \rightarrow {}^{4}T_{2g}(G)$ at ≈ 540 nm and ${}^{6}A_{1g} \rightarrow {}^{4}A_{1g}, {}^{4}E_{g}(G)$ at ≈ 450 nm).²¹

All the compounds were paramagnetic. The magnetic moments were calculated: $\mu = 6.30 \ \mu_{\rm B}$ for I, $\mu = 5.93 \ \mu_{\rm B}$ for II and $\mu = 6.80 \ \mu_{\rm B}$ for III. The experimental values of the magnetic moments were lower than the theoretical ones ($\mu = 8.53 \ \mu_{\rm B}$). The difference can be explained by antiferromagnetic interactions between the metal ions.

In order to establish the optimal conditions for the conversion of polynuclear complex compounds into spinelic lithium ferrites, their thermal decompositions were investigated by of thermogravimetry. The thermal behaviors of the complex compounds **I**, **II** and **III** are presented in Fig. 1.

Based on thermogravimetric analysis and complementary measurements (IR spectra), the following decomposition pathways may be assumed for these compounds:

 $(NH_{4})_{2}[Li_{0.5}Fe_{2.5}(C_{4}H_{4}O_{5})_{3}(OH)_{4}(H_{2}O)_{2}] \cdot 4H_{2}O(I) \xrightarrow{40-160 \ ^{\circ}C} \rightarrow (NH_{4})_{2}[Li_{0.5}Fe_{2.5}(C_{4}H_{4}O_{5})_{3}(OH)_{4}(H_{2}O)_{2}] \xrightarrow{160-245 \ ^{\circ}C} \rightarrow [Li_{0.5}Fe_{2.5}(CO_{3})_{2.5}O_{1.5}] \xrightarrow{245-570 \ ^{\circ}C} Li_{0.5}Fe_{2.5}O_{4}$

 $(\text{NH}_{4})_{6}[\text{Li}_{0.5}\text{Fe}_{2.5}(\text{C}_{4}\text{H}_{4}\text{O}_{6})_{3}(\text{OH})_{8}] \cdot 2\text{H}_{2}\text{O}(\mathbf{H}) \xrightarrow{40-140 \,^{\circ}\text{C}} \\ (\text{NH}_{4})_{6}[\text{Li}_{0.5}\text{Fe}_{2.5}(\text{C}_{4}\text{H}_{4}\text{O}_{6})_{3}(\text{OH})_{8}] \xrightarrow{140-237 \,^{\circ}\text{C}} \\ [\text{Li}_{0.5}\text{Fe}_{2.5}(\text{C}_{2}\text{O}_{4})_{4}] \xrightarrow{237-285 \,^{\circ}\text{C}} [\text{Li}_{0.5}\text{Fe}_{2.5}\text{O}_{3}(\text{CO}_{3})] \xrightarrow{285-370 \,^{\circ}\text{C}} \text{Li}_{0.5}\text{Fe}_{2.5}\text{O}_{4}$

 $(NH_4)_2[Li_{0.5}Fe_{2.5}(C_6H_{11}O_7)_3(OH_7)]$ (III) $\xrightarrow{400 \text{°C}} Li_{0.5}Fe_{2.5}O_4$



Fig. 1. Thermal analytical curves (TG, DTG and DTA) of the polynuclear complex compounds:

 $\begin{array}{l} (NH_{4})_{2}[Fe_{2.5}Li_{0.5}(C_{4}H_{4}O_{5})_{3}(OH)_{4}(H_{2}O)_{2}]\cdot 4H_{2}O\\ (a), \quad (NH_{4})_{6}[Fe_{2.5}Li_{0.5}(C_{4}H_{4}O_{6})_{3}(OH)_{8}]\cdot 2H_{2}O\\ (b) \mbox{ and } (NH_{4})_{2}[Fe_{2.5}Li_{0.5}(C_{6}H_{11}O_{7})_{3}(OH)_{7}]\ (c). \end{array}$

Characterization of the lithium ferrites

The XRD patterns of the mixed oxides obtained by thermal decomposition of the precursors, at ≈ 570 °C for I and ≈ 400 °C for II and III, indicated very poor crystallinity. In order to increase the crystallinity of the samples, two values of annealing temperature were chosen: 700 °C and 800 °C. After annealing at a temperature of 700 °C for 1 h and slow cooling, α -Li_{0.5}Fe_{2.5}O₄ was formed; all the required peaks were present in the diffraction pattern. No secondary phases were detected in the pattern of the sample obtained by the thermal decomposition of GINGASU et al.

compound II (Fig. 2a). For the other two samples obtained by decomposition of I and III, the spinel phase $Li_{0.5}Fe_{2.5}O_4$ was impure and contained also α -Fe₂O₃. Further increasing of the annealing temperature to 800 °C resulted in an augmentation of α -Fe₂O₃ in these two samples and the appearance of α -Fe₂O₃ in the sample obtained by decomposition of compound II (Fig. 2b).

According to previously published results,^{8–10,14} the single lithium ferrite phase obtained by decomposition of compound **II** annealed at 700 °C for 1 h, followed by slow cooling to room temperature showed the "ordered" (P4₁32) spinel structure, *i.e.*, α -Li_{0.5}Fe_{2.5}O₄. The average crystallite size was between 30–40 nm.

Scanning electron micrographs of the lithium ferrite obtained by thermal decomposition of compound \mathbf{II} , at different resolutions, indicate that this material tended to agglomerate because the particles were very small (Fig. 3).



Fig. 2. X-Ray diffraction patterns of Li_{0.5}Fe_{2.5}O₄, obtained by thermal decomposition of (NH₄)₆[Fe_{2.5}Li_{0.5}(C₄H₄O₆)₃(OH)₈]·2H₂O at: a) 700 °C and b) 800 °C.

SYNTHESIS OF LITHIUM FERRITES



Fig. 3. SEM of the lithium ferrite obtained by thermal decomposition of (NH₄)₆[Fe_{2.5}Li_{0.5}(C₄H₄O₆)₃(OH)₈]·2H₂O.

To confirm the formation of the spinelic phase, the IR spectra of these samples were recorded. All the spectra exhibit four bands corresponding to the four IR active fundamentals inferred by group theory considerations. These bands belong to the same representation, T_{1u} , and appear in the following regions of the spectra: v_1 (630–560 cm⁻¹), v_2 (525–390 cm⁻¹), v_3 (380–335 cm⁻¹) and v_4 (255–170 cm⁻¹).^{5,16,22–24}

The v_1 band may be attributed to vibrations of the MO₆ octahedral, the v_2 and v_3 are assigned to complex vibrations involving both octahedral and tetrahedral sites, and the v_4 band is assigned to a vibration of the tetrahedral sublattice. The infrared-active modes belong to the same symmetry T_{1u} .⁶

The presence in the IR spectra of bands at $\approx 590, 550, 470 \text{ cm}^{-1}$ with shoulders at $\approx 700 \text{ and} \approx 680 \text{ cm}^{-1}$ substantiate the formation of the "ordered" spinelic phase.^{6,25}

The magnetization *versus* H/T for lithium ferrite obtained by thermal decomposition of compound **II** annealed at 700 °C for 1 h is presented in Fig. 4.



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The value of the saturation magnetization ($\approx 50 \text{ emu g}^{-1}$) is higher than that of the same sample annealed at 800 °C for 1 h. The presence of α -Fe₂O₃ impurities lowered the magnetization of the sample. The magnetization value $\approx 50 \text{ emu g}^{-1}$ is still lower than that obtained for bulk lithium ferrite, in accordance with literature data,²⁶ due to the nanostructural character of the ferrite.

The hysteresis loops for the lithium ferrite obtained by thermal decomposition of compound **II** and annealed at 800 °C for 1 h are given in Fig. 5. These indicate that the lithium ferrite is a soft magnetic material, revealing minimal hysteresis.²⁶ It can also been observed from Fig. 5 that the loops deviate from rectangularity. The shape and width of the loop depend not only on the chemical composition and the thermal treatment but also on the particle size.²⁷ The width of the loop decreases with decreasing particle size. The value of coercive field is very small and approximately constant with temperature.



Fig. 5. The hysteresis loop for the lithium ferrite obtained by thermal decomposition of (NH₄)₆[Fe_{2.5}Li_{0.5}(C₄H₄O₆)₃(OH)₈]·2H₂O at: a) 4.0 K and b) 300 K.

SYNTHESIS OF LITHIUM FERRITES

CONCLUSIONS

Thermal decomposition of polynuclear iron–lithium complex compounds containing as ligands carboxylate anions was found to be a promising method for obtaining nanoferrite α -Li_{0.5}Fe_{2.5}O₄.

The formation temperature of α -Li_{0.5}Fe_{2.5}O₄.from the malate complex is 570 °C, while for the tartarate and gluconate compounds it is 400 °C.

Single phase α -Li_{0.5}Fe_{2.5}O₄.was obtained by thermal decomposition of compound II at 700 °C for 1 h, followed by slow cooling. The α -Li_{0.5}Fe_{2.5}O₄.phases obtained by thermal decomposition of compound I and III were impure and contained α -Fe₂O₃.

All the obtained α -Li_{0.5}Fe_{2.5}O₄ phases were nanostructured with a size smaller than 100 nm. Due to this nanostructural character, the value of the magnetization was lower than that obtained for bulk lithium ferrite.

Acknowledgement. The authors acknowledge the Ministry of Education and Research (Romania) for financial support (Research Excellence Project – Cex D11 – 17/2005-2008, "From molecular and supramolecular multimetallic complexes to novel magnetic materials").

ИЗВОД

СИНТЕЗА ЛИТИЈУМ-ФЕРИТА ИЗ ПОЛИМЕТАЛНИХ КАРБОКСИЛАТА

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Литијум-ферит је добијен термичком разградњом три полинуклеарна комплексна једињења која као лиганде садрже анјоне малеинске, винске и глуконске киселине: $(NH_4)_2[Fe_{2.5}Li_{0.5}(C_4H_4O_5)_3(OH)_4(H_2O)_2]\cdot 4H_2O$ (I), $(NH_4)_6[Fe_{2.5}Li_{0.5}(C_4H_4O_6)_3(OH)_8]\cdot 2H_2O$ (II) и $(NH_4)_2[Fe_{2.5}Li_{0.5}(C_6H_{11}O_7)_3(OH)_7]$ (III). Полинуклерна комплексна полазна једињења окарактерисана су хемијском анализом, IR и UV–Vis спектрима, магнетним мерењима и термогравиметријском анализом. Добијени литијум-ферит окарактерисан је методама дифракције х-зрака и скенирајуће електронске микроскопије, као и IR спектрима и магнетним мерењима. Регистровано је да је добијена једино α -Li_{0.5}Fe_{2.5}O₄ фаза термичком разградњом тартаратног комплекса на температури 700 °C током 1 h. Вредност магнетизације од око 50 еmu g⁻¹ мања је од оне добијене за компактни литијум-ферит због наноструктурних карактеристика синтетисаног ферита. Величина честица мања је од 100 nm.

(Примљено 6. марта, ревидирано 20. маја 2008)

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J. Serb. Chem. Soc. 73 (10) 989–995 (2008) JSCS–3780 JSCS@tmf.bg.ac.yu • www.shd.org.rs/JSCS UDC 537.872+539.124:547.686:54.022+541.5 Original scientific paper

The Hall rule in fluoranthene-type benzenoid hydrocarbons

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(Received 20 May 2008)

Abstract: The applicability of the Hall rule (linear relation between the total π -electron energy and the number of Kekulé structures) was investigated in the case of fluoranthene-type benzenoid hydrocarbons. It was found that the originnal Hall rule is not obeyed, but holds for sets of isomers with a fixed number of bay regions. For such groups of isomers, two apparently contradictory Hall-type rules were conceived, and it was found that both give almost identical numerical results.

Keywords: Hall rule; total π -electron energy; Kekulé structures; fluoranthene; benzenoid hydrocarbons; fluoranthene-type hydrocarbons.

INTRODUCTION

The Hall rule, discovered by George Hall in the $1970s^1$ and formulated in a quantitative manner in the 1980s,^{2,3} claims that within a set of isomeric benzenoid hydrocarbons, the total π -electron energy, E, increases in a linear manner with the number of Kekulé structures, K. Later studies^{4–9} showed that the Hall rule holds only for benzenoid molecules of small and moderate size (with up to 9 hexagons) and is violated in the case of larger systems. Recently a modified version of the Hall rule was proposed,¹⁰ in which the dependence of E on K is not strictly linear.

Details of the theory of benzenoid hydrocarbons, including the Hall rule, can be found in a book,¹¹ review,¹² and the references cited therein.

Whereas benzenoid hydrocarbons have been in the focus of interest of theoretical chemists for more than a century (see the recent papers^{13–18} and the references quoted therein), a very similar class of conjugated molecules has almost completely evaded their attention. These are the fluoranthene-type benzenoids, obtained by condensing two benzenoid fragments *via* a five-membered (cyclopentadiene) ring. Four representatives of the fluoranthene-type benzenoid family are depicted in Fig. 1, together with a diagram indicating their general structure.

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doi: 10.2298/JSC0810989D



Fig. 1. Fluoranthene (1) and three other members of the family of fluoranthene-type benzenoid molecules (2, 3, 4). Diagram F shows the general structure of a fluoranthene-type system: two benzenoid fragments (X and Y) are joined *via* a five-membered ring. The arrows point to edges, the count of which is the number of bay regions, *B*; thus B(1) = 2, B(2) = 7, B(3) = 7 and B(4) = 8.

Whereas scores of papers and books are devoted to the theoretical chemistry of benzenoid hydrocarbons, the first article in which fluoranthene-type benzenoids were systematically investigated was published only quite recently.¹⁹ Almost nothing is known on the topological and structure–property relations of fluoranthene-type benzenoid molecules. The aim of the present work (as well as of studies that we intend to accomplish in the future) is to contribute towards filling this hitherto overseen gap in the theory of polycyclic conjugated molecules.

A PROBLEM RELATED WITH THE HALL RULE

According to the Hall rule, if X is a benzenoid molecule with *h* hexagons, $h \le 9$, then its total π -electron energy E(X) and Kekulé structure count K(X) are (as a good approximation) related as:^{2,3}

$$E(\mathbf{X}) = aK(\mathbf{X}) + b$$

where *a* and *b* are constants that depend on *h*.

Now, a fluoranthene F is composed of two benzenoid fragments X and Y (*cf.* Fig. 1) and its total π -electron energy is up to an additive constant equal to E(X) + E(Y). On the other hand, the Kekulé structure count of F is equal to K(X)K(Y). Therefore, one arrives at the following seemingly contradictory Expressions (1) and (2):

1. If the Hall rule is applicable to the entire fluoranthene, then:

$$E(F) = a_1[K(X)K(Y)] + b_1$$
(1)

2. Since the Hall rule is applicable to the benzenoid fragments X and Y, it has to be:

$$E(F) = a_2[K(X) + K(Y)] + b_2$$
(2)

In the above (approximate) formulas, a_1 , b_1 , a_2 and b_2 are pertinently chosen constants (see below). In Eq. (2), for the sake of simplicity, it is assumed that the fragments X and Y have an equal number of hexagons.

In order to decide which of the two Hall-type relations hold, Eq. (1) or Eq. (2), a detailed numerical study was undertaken.

NUMERICAL WORK

In order to test the validity of Eqs. (1) and (2), a data set was constructed consisting of all possible fluoranthene isomers in which both fragments X and Y are tetracyclic catacondensed benzenoids, a total of 290 isomers (two of these are compounds **2** and **3** in Fig. 1). Five tetracyclic catacondensed benzenoids exist, *i.e.*, naphthacene (K = 5), benzo[a]anthracene (K = 7), chrysene (K = 8), benzo-[c]phenanthrene (K = 8), and triphenylene (K = 9). Therefore, the Kekulé structure counts of the considered fluoranthene isomers assume one of the following nine values: 25, 35, 40, 45, 49, 56, 63, 64, and 72. (There are no isomers with $K = 9^2 = 81$ because triphenlyene cannot be the X-fragment of a fluoranthene-type benzenoid molecule).

The correlation between the total π -electron energy (*E*) and the Kekulé structure count (*K*) of the considered fluoranthene isomers is shown in Fig. 2. At the first glance, the correlation is prohibitively bad.



Fig. 2. The total π -electron energies (*E*) of isomeric fluoranthenes *vs.* their Kekulé structure counts (*K*). It is easily seen that in addition to *K*, there is at least one more structural feature that influences *E*. This structural feature was recognized as the number of bay regions, *B* (see Fig. 1).

A detailed inspection of the data shown in Fig. 2 indicates that some structural parameter other than the Kekulé structure count influences the value of E, and that its effect is of a similar magnitude as that of the Kekulè structures. It was not to difficult to identify this structural feature, since an analogous problem was a considered long time ago and solved within the theory of benzenoid hydrocarbons.^{11,20}

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On the perimeter of a benzenoid of a fluoranthene-type system, there are bonds between carbon atoms to which no hydrogen atoms are attached. In the terminology of molecular graphs, on the perimeter there are edges connecting two vertices of degree 3. The number of such carbon–carbon bonds (*i.e.*, edges) is called "the number of bay regions" and is denoted by *B*. Examples illustrating the bay-region concept are to be found in Fig. 1.

It was established²⁰ that within isomeric benzenoid hydrocarbons with a fixed number of Kekulé structures, the total π -electron energy depends in a nearly linear (increasing) manner on the parameter B. Theoretical considerations¹⁹ (based on the structure-dependence of the spectral moments) suggest that an analogous regularity should be expected also in the case of fluoranthenes. That this indeed is the case is seen from Fig. 3 and the data collected in Table I.



Fig. 3. The total π -electron energies (*E*) of isomeric fluoranthenes possessing B = 7 bay regions *vs.* their Kekulé structure counts (*K*). The relatively good quality of this correlation confirms Eq. (1); the statistical data for this correlation and those for other values of *B* are given in Table I.

TABLE I. Data showing the quality of the correlation between the total π -electron energy and the two different counts of Kekulé structures, Eqs. (1) and (2), for isomeric fluoranthene-type benzenoid hydrocarbons with a fixed number of bay regions (*B*); *N.I.*: number of isomers, *R*: correlation coefficient. The correlations for *B* = 7 are shown in Figs. 3 and 4.

В	N.I.	<i>R</i> (Eq. (1))	<i>R</i> (Eq. (2))
2	1	_	_
3	2	_	_
4	20	0.9802	0.9861
5	59	0.9938	0.9955
6	93	0.9939	0.9941
7	79	0.9953	0.9947
8	36	0.9948	0.9955

However, when an analogous analysis is performed by plotting *E* not *versus* K = K(X)K(Y) but *versus* K(X) + K(Y), almost identical results are obtained, see Fig. 4 and Table I.

The way in which the coefficients a_1 and b_1 in Eq. (1) depend on the number of bay regions is shown in Fig. 5. The dependence of a_2 and b_2 in Eq. (2) is ana-

logous. Other statistical data on the Correlations (1) and (2) can be obtained from the authors, upon request.



Fig. 5. Dependence of the coefficients a_1 and b_1 in Eq. (1) on the parameter *B*. The *B*-dependence of the coefficients a_2 and b_2 in Eq. (2) is almost identical and is therefore not shown.

DISCUSSION AND CONCLUDING REMARKS

The main difference between benzenoid- and fluoranthene-type systems is in the role of the bay regions. The effect of bay regions in fluoranthene-type hydrocarbons is much more pronounced and, therefore, only isomers with equal *B*-values may be compared. Thus, whereas in the case of benzenoid hydrocarbons, the Hall rule applies to all isomers irrespective of their *B*-values, in fluoranthenes the Hall-type regularities (either Eq. (1) or Eq. (2)) are valid only within groups of isomers with a fixed number of bay regions. If fluoranthene isomers with non-equal *B*-values are considered, then the correlation between *E* and *K* is very weak (yet linear), as seen in Fig. 2.

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From the data presented in the preceding section, one arrives at a somewhat unexpected conclusion: Both forms of the Hall rule, the approximate Formulas (1) and (2), have equal precision and their accuracy is practically identical. This, in turn, means that the original Hall rule can be applied with similar success to both the entire fluoranthene molecules and the two benzenoid fragments that form these molecules. Although the mathematical form of the two Hall rules appears to be significantly different and not mutually consistent, the quality of their numerical performance is indistinguishable.

In summary, it was shown in this paper that in the case of fluoranthene-type benzenoid hydrocarbons, the original Hall rule is not applicable. However, under certain restricted conditions (*e.g.*, fixed *B*-values) regularities similar to the Hall rule could be established. These results indicate that the single five-membered ring in the fluoranthene molecule drastically perturbs the regular π -electron configuration found in ordinary benzenoid hydrocarbons.

ИЗВОД

НАLL-ОВО ПРАВИЛО У БЕНЗЕНОИДНИМ УГЉОВОДОНИЦИМА ФЛУОРАНТЕНСКОГ ТИПА

ЈЕЛЕНА ЂУРЂЕВИЋ, СЛАВКО РАДЕНКОВИЋ и ИВАН ГУТМАН

Природно-машемашички факулшеш Универзишеша у Крагујевцу, Крагујевац

Испитивана је применљивост Hall-овог правила (линеарне релације између укупне π-електронске енергије и броја Kekulé-ових структура) у случају бензеноидних угљоводоника флуорантенског типа. Нађено је да оригинално Hall-ово правило није задовољено, али да важи за групе изомера са фиксираним бројем залива. За такве групе изомера, дошло се до два наизглед контрадикторна правила Hall-овог типа, и нађено је да оба дају готово идентичне нумеричке резултате.

(Примљено 20. маја 2008)

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J. Serb. Chem. Soc. 73 (6) 997–1009 (2008) JSCS–3781 JSCS@tmf.bg.ac.yu • www.shd.org.rs/JSCS UDC 546.47–31:544.478:66.094.258+547.264 Original scientific paper

Characterization of various zinc oxide catalysts and their activity in the dehydration-dehydrogenation of isobutanol

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(Received 24 Novembar 2007, revised 23 January 2008)

Abstract: Mono and bifunctional zinc oxide catalysts were prepared to study their activity in the catalytic conversion of isobutanol. The prepared catalysts were characterized by X-ray diffraction analysis and nitrogen adsorption measurements. The conversion of isobutanol was taken as a model reaction to measure the catalytic activity of the prepared oxide catalysts. The overall conversion yield of competitive dehydration–dehydrogenation reactions of isobutanol was found to be higher over the binary oxide catalysts than over the single oxide one at all reaction temperature within the range 250–400 °C. The binary oxide catalysts were more selective for isobutene, as the predominant dehydrated product. The single oxide catalyst exhibited higher selectivity towards isobutyraldehyde as the dehydrogenated product, and the selectivity increased with increasing reaction temperature.

Keywords: metal oxides; acid-base catalysis; isobutanol conversion.

INTRODUCTION

Recently the United States Environmental Protection Agency announced a proposal to reduce the permissible lead level in U.S. leaded gasoline by 91 %. The importance of dehydration of higher alcohols, more than C_2 , lies in the preparation of isobutene, which is one of the radiant for the production of gasoline octane enhancers. In addition, intermediate compounds for fine chemical industries, as 1,2-diphenylethanol, are produced.

Macho *et al.*¹ used alumina in the dehydration of C₄ alcohols whereby positional and skeletal isomerization of formed C₄ alkenes occurred in a fixed bed reactor operated under atmospheric pressure and at moderate reaction temperatures 300-470 °C.

Mixed oxides, such as ZrO₂/SiO₂^{2,3} or ZnO/Al₂O₃, modified with 23 ppm lithium, of different compositions were prepared by the sol–gel method.⁴ The catalysts exhibited high catalytic activity towards isopropanol dehydration, with a

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selectivity of 100 %. Vaidya *et al.*⁵ studied the dehydration of 1,4-butanediol to tetrahydrofurane using a strong acid cation exchange resin as the catalyst and found that the produced water adsorbed on the active resin inhibited the dehydration reaction.

Catalysts based on Keggin-type heteropoly acids supported on silica and tubular ceramic silica membrane^{6,7} were used for the dehydration of alcohol.

A series of metal phosphates (Al, Fe, Ni and Mn) were prepared by the ammonia gelation method and used in the competitive dehydration–dehydrogenation reaction of cyclohexanol. The results proved that the dehydration was realized not only on acid sites, but also on basic sites.⁸

The objective of this work was to study the structural phase changes accompanying the preparation of zinc oxide and binary zinc oxide catalysts. The catalytic behavior of the prepared zinc oxide catalyst and the role of adding TiO_2 and Cr_2O_3 on the performance of zinc oxide catalyst towards the isobutanol conversion reaction were studied.

EXPERIMENTAL

Preparation of the catalysts

Three different oxide catalysts: zinc oxide, zinc oxide-titanium oxide and zinc oxide-chromium oxide, all mixed with bentonite, were prepared.

The zinc oxide–bentonite catalyst (Z) was prepared by mixing an equal weight ratio of zinc oxide and acid activated bentonite (activated with 2 % HCl).

The zinc oxide–titanium oxide–bentonite catalyst (ZT) was prepared by mixing zinc oxide, titanium oxide and acid-activated bentonite at a weight ratio of 1.5:1:2.5, respectively, whereas the zinc oxide–chromium oxide–bentonite catalyst (ZC) was prepared *via* the mixing of hydrated chromium oxide (freshly precipitated from an aqueous solution of chromium chloride using an ammonia solution with subsequent washing and drying) with zinc oxide and acid activated bentonite at a weight ratio 1.5:1:2.5, respectively.

The prepared catalysts were dried at 120 °C for two hours with continuous agitation and then ground to mesh size 0.16 mm. Subsequently, the catalysts were calcined at a temperature of 750 °C for 12 h in presence of purified dry air to produce Z*, ZT* and ZC*.

Catalysts characterization

The structural changes of the prepared catalysts (zinc oxide, zinc oxide–titanium oxide and zinc oxide–chromium oxide–bentonite) were investigated by X-ray diffraction analysis (XRD) using a Shimadzu XD-D1-X-ray diffraction apparatus, equipped with a monochromator for CuK α radiation to determine their crystallization behavior. The apparatus was provided with a software program which allowed the changes in the crystallite size of the mono- and bi-metal oxide catalysts to be determined.

Nitrogen adsorption–desorption measurements were conducted at -196 °C using a Quanta Chrome Nova 2000 instrument. The samples were out-gassed (10^{-4} Pa) at 300 °C and the surface areas were calculated from the adsorption curves by the BET method.

Catalytic activity

The conversion reaction of isobutanol was taken as a model reaction for measuring the catalytic activity of the prepared catalysts. The isobutanol catalytic reaction was performed in

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a pulse micro-catalytic reactor operated under atmospheric pressure at reaction temperatures ranging from 250–400 °C. The micro-reactor (diameter = 0.20 mm and length = 15 cm) was charged with 500 mg of dried catalyst. A 2.0 μ l dose of isobutanol was injected into a nitrogen stream flowing continuously down the catalyst bed at a rate of 40 ml min⁻¹. The reaction outputs were immediately analyzed by flame ionization detector (FID) through a chromatographic column (silicon oil-550) directly attached to the reactor. The column was packed with chromosorb b of 80–100 mesh size.

RESULTS AND DISCUSSION

Catalysts characterization

X-ray diffraction analysis. The X-ray diffraction patterns for all the prepared catalysts are presented in Figs. 1–3. The diffraction pattern for acid-activated bentonite reveals the appearance of sharp and high intensity diffraction lines, characteristic for bentonite material (ASTM 03-0010).



Fig. 1. X-Ray diffraction patterns for (a) bentonite, (b) Z- and (c) Z*-catalyst.

For the Z-catalyst (Fig. 1b), new diffraction lines with high intensity appeared which are characteristic for the ZnO phase (ASTM 05-0664). In addition, the lamella line of the bentonite was shifted to the left (to a higher *d*-spacing), which evidenced the penetration of ZnO to inside the bentonite lamella resulting in this expansion. The diffraction pattern for the calcined Z-catalyst, Z^* (Fig. 1c), showed SAAD and RIAD

the presence of a zinc silicate phase, according to ASTM (79-2005). The formation of zinc silicate actually arose from the interaction of zincite (a defected form of calcined ZnO which has the ability to trap electrons) with the bentonite structure during the calcination step at 750 $^{\circ}$ C.⁹



Fig. 2. X-Ray diffraction pattern for: (a) ZTand (b) ZT*-catalysts. Fig. 3. X-ray diffraction pattern for: (a) ZCand (b) ZC*-catalysts.

The diffractogram for the ZT catalyst (Fig. 2a) evidenced the presence of the anatase form of TiO₂ (ASTM 09-0240),¹⁰ in addition to the characteristic ZnO lines. The characteristic bentonite lines were still visible but with a low intensity, which indicates the insertion of both ZnO and TiO₂ into the bentonite lamella.

For the calcined ZT-catalyst, ZT* (Fig. 2b), new phases were detected, *i.e.*, the spinel crystalline structure Zn_2TiO_4 and/or the cubic $Zn_2Ti_3O_8$ (ASTM 13-0471), besides the hexagonal-coordinated ZnTiO₃ (ASTM 15-0591).

The X-ray diffraction pattern for the ZC-catalyst (Fig. 3a) revealed the disappearance of the main characteristic bentonite lines and a significant decrease in the intensity of the main ZnO lines. This indicates that the Cr_2O_3 particles may block the bentonite lamella and are probably trapped in the ZnO lattice.

For the calcined ZC-catalyst, ZC*, the intensity of the main ZnO lines sharply increased, in addition to the appearance of new lines at 2θ angles: 29.9 and 36.5° (Fig. 3b), which are related to the formation of the spinel crystalline structure ZnCr₂O₄ which favors catalyst dispersion.¹¹

Crystallite size. The crystallite sizes for bentonite, as well as for the prepared and calcined catalysts, are summarized in Table I.

The data for the Z-catalyst shows that the calculated crystallite size decreesed from 27.5 to 17.1 nm on mixing ZnO with bentonite. This result suggests the

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dispersion of ZnO particles into the bentonite lamella, in agreement with XRD data. Moreover, the interaction of the Z-catalyst with titanium oxide caused a decrease in the crystallite size, confirming that the TiO₂ species preserved the high dispersion of the ZnO particles and preventing them from growing. The reverse was the case after mixing the Z-catalyst with chromium oxide, *i.e.*, an increase in crystallite size was observed, indicating that the chromium oxide crystallites were larger than the titanium dioxide ones.

TABLE I. Crystallite size, nm, for the studied calcined catalysts

Position, Å	Bentonite	Ζ	Z*	ZT	ZT*	ZC	ZC*
14.46	27.5	17.1	_	9.1	-	_	-
3.33	179.5	141.3	_	157.3	-	193.1	-
2.60	_	199.1	180.5	151.1	227.5	203.9	261.8

The calcinations of the Z-catalyst (Z^*) resulted in a decrease in the crystallite size, which is an indication for the formation of smaller "zinc silicate" crystallites, as verified by X-ray diffraction analysis.

The crystallite size increased, however, after calcination of the ZT and ZC catalysts (ZT* and ZC*). This increase is due to the formation of zinc, titanium and chromium binary oxides upon calcination, which may have larger crystallite sizes.

Nitrogen adsorption technique. The different surface characteristics, *i.e.*, specific surface area (S_{BET}), total pore volume (V_p) and average pore radius (r_H), for the studied calcined catalysts were determined from nitrogen adsorption isotherms conducted at -196 °C and the results are given in Table II.

TABLE II. Surface properties of the studied calcined catalysts

Catalyst	$S_{\rm BET}$ / m ² g ⁻¹	$V_{\rm p}$ / ml g ⁻¹	$r_{ m H}$ / Å
Z*	6.8	0.0056	16.5
ZT*	10.6	0.0090	17.0
ZC*	10.4	0.0086	16.5

The BET surface area for the Z*-catalyst increased from 6.8 to $\approx 10.5 \text{ m}^2 \text{ g}^{-1}$ upon interaction of the zinc catalyst with chromium or titanium oxide, which resulted from the repulsive forces induced by the anionic sites "O" that aided in the dispersion of the particles and consequently the surface area increased.¹² Moreover, the increase in the surface area was accompanied with an increase in the pore volume without an increase in the pore radius (Table II), confirming the intrusion of chromium and titanium oxide phases inside the zinc oxide pores.

Catalytic activity

The catalytic activity and selectivity of the prepared catalysts in the competitive dehydration–dehydrogenation reaction of isobutanol were investigated at reaction temperatures in the range from 250–400 °C. The data is illustrated graphically in Fig. 4.





For all the oxide catalysts, the total conversion increased continuously with increasing reaction temperature. Furthermore, the binary oxide catalysts (ZT* and ZC*) exhibited a higher conversion at all reaction temperatures, reaching near complete conversion at 400 °C compared with the zinc oxide catalyst (Z*).

The converted products mainly consisted of isobutene, as the dehydrated product, and isobutyraldehyde, as the dehydrogenated product. The yield of isobutene over all catalysts increased gradually with increasing reaction temperature, whereas considerable higher values were observed over the binary oxide (ZC* and ZT*) catalysts than over the zinc oxide catalyst (Fig. 4).

Meanwhile, the yield of isobutyraldehyde was similar at lower reaction temperatures, while drastic increase in the yield was obtained using the Z^* catalyst. The difference in the activity of zinc oxide and binary oxide catalysts seems to depend on the surface electronegative character of each catalyst. The effect of surface electronegativity on the activity was interpreted in term of the relative abundance of basic and acidic sites required for the chain growth pathway.¹³

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Metal oxides are composed of a metal and oxygen and are consequently characterized by an almost ionic bond. The coordination of ions at an ideal surface is, by necessity, incomplete with respect to the bulk.¹⁴ Thus, the partially uncoordinated metal cations and oxide anions lying at the surface of metal oxide crystals can act as acids and bases, respectively. Hence, Lewis acid sites (coordinatively unsaturated cations) and basic sites are expected and found at ionic metal oxides. These coordinatively unsaturated cations exposed at the surface of ionic oxide can, consequently, interact with basic molecules forming a new coordination bond, thus completing or increasing the overall coordination at the surface cations.

Accordingly, the highest conversion of isobutanol was obtained with ZT* then ZC* and finally Z*, which could be attributed mainly to the difference in the acid–base feature of each oxide catalyst taking into account the incorporation of highly electronegative cations "Lewis acids", such as Ti^{4+} and Cr^{3+} . A highly Lewis acidic surface enhances the approach of basic reactants to the active site and consequently strong Lewis interaction with metal cations. Thus, the moderately reducible oxide catalyst (Z*) exhibits a lower conversion than the mixed oxide catalysts (Fig. 4).

Hence, the high activity of all the prepared oxide catalysts with increasing reaction temperature could be explained based on redox mechanisms.

On the other hand, the polarizing power (charge to ionic radii ratio) may also affect the overall conversion rate. The stronger are the polarizing power of an acidic Lewis cation and the basic strength of an adsorbate, the stronger is the Lewis interaction, *i.e.*, the greater is the Lewis strength of the surface sites. Thus, incorporation of Ti^{4+} and Cr^{3+} , which have a polarizing power of 6.6 and 4.9, respectively, should enhance the Lewis interaction with isobutanol molecules, compared with Zn^{2+} which has a polarizing power of 3.3.¹⁵

Consequently, the considerable increase in the yield of isobutene over the binary oxide catalysts comparing with the single oxide catalyst (Fig. 4) may be attributed to the additional Lewis acidity arising from the presence of Cr^{3+} or Ti^{4+} on the oxide surface, which is expected to enhance the dehydration reaction.

Meanwhile, the observed divergence in the yield of isobutyraldehyde at higher reaction temperatures (350–400 °C) in the ranking $Z^* > ZT^* > ZC^*$ (Fig. 4) could be explained on the basis of the reducing character on the surface of the catalysts. The lower density of exposed oxidizing centers (Lewis sites) on the surface of the ZnO catalyst compared with the binary oxides (ZT* and ZC*) and enrichment with oxygen anions seems to be responsible for the suppression of the dehydration reaction and the enhancement of the abstraction of the more acidic α -H from the alkoxy intermediates (see reaction mechanism), which gives rise to the abrupt increase in the yield of isobutyraldehyde.

On the other hand, the selectivity of the prepared oxide catalysts towards both dehydration (isobutene) and dehydrogenation (isobutryaldehyde) products SAAD and RIAD

as represented by the histograms shown in Fig. 5, revealed that the selectivity to isobutene formation is greatly enhanced over the binary oxide catalysts (ZC* and ZT*), indicating a significant increase at higher reaction temperatures (350-400 °C). Moreover, the catalyst ZC* exhibited higher and relatively similar values of isobutene selectivity in comparison with the ZT* catalyst. Conversely, the Z* catalyst showed the highest selectivity to isobutyraldehyde formation, which was constant at all reaction temperatures. The selectivity of the binary oxide catalysts decreased gradually with increasing reaction temperature, revealing a higher selectivity to isobutyraldehyde formation over the ZT* catalyst.



The reversible selectivity trends for the prepared mono and binary oxide catalysts may be expressed due to a low oxygen supply resulting from the lower reduction potential of the Ti and Cr species (*i.e.*, a high attraction to oxygen), which limited the dehydrogenation reaction and enhanced the concerted attack by isobutanol molecules through Lewis acid–Brönsted base pair site ($T^{4+}O^{2-}$ and $Cr^{3+}O^{2-}$) of balanced strength.

However, dehydration is always developed in any solid in a concerted way but separately in every acid or base site (Figs. 6 and 7) through two steps of adsorption and desorption. $^{16-18}$

Adsorption Step



Concerted desorption step



Fig. 6. Reaction mechanism of isobutanol dehydration to isobutene on surface acid sites *via* the concerted mechanism.

Adsorption step



Concerted desorption step



Fig. 7. Reaction mechanism of isobutanol dehydration on a basic site via the concerted mechanism.

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Reaction mechanism of isobutanol conversion

From a general point of view, dehydration of an alcohol to an olefin on single oxide and binary oxide catalysts can proceed through two different elimination mechanisms, E_{ICB} and E_2 .

Accordingly, the dehydration of isobutanol on the prepared metal oxide catalysts can proceed through the two-elimination mechanisms, E_{ICB} and E_2 , as shown in Scheme 1.¹⁹ The E_{ICB} pathway involve a surface alkoxy intermediate on both a strongly basic site and a weak Lewis acid site ($Zn^{2+}O^{2-}$ pairs). Formation of the olefin takes place by β -H elimination from the carbanion intermediate. Thus, ZnO contains strong basic sites consisting of O^{2-} anions, which catalyze alcohol dehydration to olefin *via* the E_{1cB} pathway. In contrast, the E_2 elimination is a single-step, concerted mechanism in which the OH group and β -H are simultaneously abstracted by a Lewis acid–Brönsted base pair site of balanced strength, such as $Ti^{4+}O^{2-}$ and $Cr^{3+}O^{2-}$ pairs. This mechanism leads to the formation of more electronegative cations (Ti^{4+} and Cr^{3+}) increases the density and strength of the acid sites and thus decreases the activation energy of β -H abstraction.²⁰ Therefore, isobutanol dehydration on mixed oxide catalysts is more likely to proceed through the E_2 elimination mechanism (Scheme 1).



Scheme 1. Mechanism of isobutene formation.
Dehydrogenation of an alcohol to an aldehyde is a typical base catalyzed reaction, Scheme 2.^{21,22}



Scheme 2. Mechanism of isobutyraldehyde formation.

Weak Lewis acid–strong Brönsted base site pairs $(Zn^{2+}O^{2-})$ with strongly basic oxygen play an important role because they are required for hydrogen abstraction leading to an alkoxy intermediate. Dehydrogenation commences with alcohol chemisorption on $Zn^{2+}O^{2-}$ site pairs which cleavage the OH bond to form a surface alkoxide intermediate bound to a Zn^{2+} acid center. The α -hydrogen in the alkoxide group is abstracted by a neighboring basic site in order to form adsorbed aldehyde. Thus, the formation of isobutyraldehyde by isobutanol dehydrogenation prevails on a basic oxide.

However, samples which have Ti or Cr surface enrichment are suggested to provide additional Lewis centers which may stabilize the alkoxy intermediates; hence Ti–O–Zn and Cr–O–Zn species may be particularly effective and abundant sites for dehydrogenation. Moreover, benefit from the presence of these species is suggested to be enhancement of H₂ dissociation and O–H or C–H activation during the dehydrogenation of isobutanol.²³

CONCLUSIONS

From the previous discussion, it may be concluded that the catalytic dehydration reaction of isobutanol to isobutene, in addition to its eminent practical interest, can be employed as a reaction model for the characterization of a solid acid base catalyst, but taking into account the dehydration reaction proceeds not only on acid sites but also on basic ones and that isobutryaldehyde, obtained through the dehydrogenation reaction, requires additional redox ability not necessary associated to basic sites.

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Acknowledgement. The authors are grateful for Prof. Dr. S. Mikhail (Refining division, Egyptian Petroleum Research Institute, Cairo) for constructive and fruitful discussion.

ИЗВОД

КАРАКТЕРИЗАЦИЈА РАЗНИХ ЦИНК-ОКСИДНИХ КАТАЛИЗАТОРА И ЊИХОВА АКТИВНОСТ ЗА ДЕХИДРАТАЦИЈУ–ДЕХИДРОГЕНАЦИЈУ ИЗОБУТАНОЛА

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Синтетисани су моно и бифункционални цинк-оксидни катализатори за потребе испитивања каталитичке конверзије изобутанола. Добијени катализатори окарактерисани су дифракцијом х-зрака и методом адсорпције азота. Реакција конверзије изобутанола узета је као модел реакција за испитивање каталитичке активности добијених оксидних катализатора. Утврђено је да је укупан принос конверзије у реакцији дехидратације/дехидрогенације изобутанола већи на катализатору од бинарног оксида него на катализатору од појединачног оксида при температурама у опсегу 250–400 °C. Катализатор од бинарног оксида је селективнији у случају изобутена као преовлађујућег производа дехидратације. Катализатор од појединачног оксида је селективнији у случају реакције дехидрогенације са изобутиралдехидом као производом реакције, а селективност се побољшава са порастом температуре реакције.

(Примљено 24. новембра 2007, ревидирано 23. јануара 2008)

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J. Serb. Chem. Soc. 73 (10) 1011–1019(2008) JSCS–3782 JSCS@tmf.bg.ac.yu • www.shd.org.rs/JSCS UDC 667.287.4:536.75+536.722:543.24 Original scientific paper

Thermodynamic characterization of the dimerization equilibrium of newly synthesized polymethine cyanine dyes

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(Received 14 September 2007, revised 16 June 2008)

Abstract: The monomer–dimer equilibrium and thermodynamics of three new cyanine dyes were investigated by spectrophotometric and chemometric methods. The dimerization constants of these new cyanine dyes were determined by studying the dependence of their absorption spectra on the temperature in the range 25–80 °C at concentrations of 3.0×10^{-4} , 1.9×10^{-4} and 1.1×10^{-4} M for dye **1**, **2** and **3**, respectively. The processing of the data, performed for the quantitative analysis of pure spectral profiles, was based on the simultaneous resolution of the overlapping bands in the whole set of absorption spectra. From the dimerization constant and its dependence on temperature, the values of the standard enthalpy change and entropy change of dimerization were calculated.

Keywords: chemometrics; dimerization; cyanine dyes; absorption titration; enthalpy; entropy.

INTRODUCTION

Dimerization of dyes in aqueous solution has been studied extensively as the most fundamental model of the self-aggregation of biological molecules and biomacromolecule–ligand interactions, 1-3 and as the basis for their application as tunable lasers and other practical uses. 4-6 Various factors have been found to affect the dimerization behavior, *i.e.*, dye structure, solvent, coexisting salt, temperature and pressure. Thermodynamic parameters, such as standard enthalpy and entropy change on dimerization, which are derived from the temperature dependence of the dimerization constant, have given some insight into the forces that maintain the dimer structure in solution.

In recent years, there has been increased interest in the field of synthesis and application as nucleic acid labels of cyanine (polymethine) dyes absorbing in different visible spectral regions. The number of both patents and scientific publica-

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tions is evidence for the commercial, scientific and practical importance of these probes in nucleic acid research, as well as clinical and environmental analysis. Representatives of this class of nucleic acid stains have fluorescence excitations and emissions covering the visible spectrum from blue to near infrared, with additional absorption peaks in the UV region, which makes them applicable in many different types of instrumentation with different light sources. Replacement of even one substituent in the dye molecule can lead to novel and sometimes better properties for nucleic acid detection.⁷ We believe that such an important research area requires more new representatives to be investigated in order to establish their new properties on interaction with nucleic acids.

Spectroscopic methods are in general highly sensitive and as such suitable for studying chemical equilibria in solution. When the components involved in the chemical equilibrium have distinct spectral responses, their concentrations can be measured directly, and the determination of the equilibrium constants is trivial.⁸

In this study, the dimerization equilibrium of newly synthesized cyanine dyes⁹ was characterized by absorption titration and chemometrics data analysis, carried out by the DATAN package developed by Kubista *et al.*^{10–15} The theory and application of the physical constraints method was discussed by Kubista *et al.* and is discussed here briefly.

Theory

Absorption spectra recorded at different temperatures are arranged as rows in an $n \times m$ matrix **A**, where *n* is the number of temperature intervals and *m* is the number of data points in each spectrum. **A** is decomposed into an orthogonal basis set using, for example, NIPALS.^{16,17}

$$\mathbf{A} = \mathbf{TP}' + \mathbf{E} \approx \mathbf{TP}' = \sum_{i=1}^{r} t_i \mathbf{p}_i$$
(1)

where t_i ($n \times 1$) are orthogonal target vectors and p_i^* ($1 \times m$) are orthogonal projection vectors. These are mathematical constructs and do not correspond to any physical property of the system. r is the number of spectroscopically distinguishable components, and **E** is the error matrix containing experimental noise, if the right value of r is selected. For a well-designed experiment, **E** is small compared to **TP**' and can be neglected. Assuming linear response, the recorded spectra are also linear combinations of the spectral responses, v_i ($1 \times m$), of the components:

$$\mathbf{A} = \mathbf{C}\mathbf{V} + \mathbf{E} \approx \mathbf{T}\mathbf{P}' = \sum_{i=1}^{r} c_i \mathbf{v}_i$$
(2)

where c_i ($n \times 1$) are vectors containing the component concentrations at the different temperatures. The two equations are related by a rotation:¹⁸

$$C = TR^{-1}$$
 (3)
 $V = RP'$ (4)

where **R** is an $r \times r$ rotation matrix. For a two-component system:

$$\mathbf{R} = \begin{bmatrix} r_{11} & r_{12} \\ r_{21} & r_{22} \end{bmatrix}$$

and

$$\mathbf{R}^{-1} = \frac{1}{\eta_1 r_{22} - \eta_{2r} r_{22}} \begin{bmatrix} \eta_1 & -\eta_2 \\ -r_{21} & r_{22} \end{bmatrix}$$
(5)

Since a single sample is studied, the total concentration must be constant, constraining the matrix $\mathbf{R}^{.19}$ For a monomer–dimer equilibrium, $2X \leftrightarrow X_2$, the total concentration of monomers is constant:

$$c_{\rm X} + 2c_{\rm X_2} = c_{\rm tot} \tag{6}$$

Combining Eq. (6) with Eq. (3), one obtains:

$$\frac{1}{r_{11}r_{22} - r_{12}r_{22}}(t_1r_{22} - t_2r_{21} - 2t_1r_{12} + 2t_2r_{11}) = c_{\text{tot}}$$
(7)

which can be written as:

$$f_{11}t_1 + f_{12}t_2 = c_{tot}$$
(8)

where

$$f_{11} = (r_{22} - 2r_{12})(r_{11} - r_{12}r_{21})^{-1}$$
(8a)

and

$$\mathbf{f}_{12} = (2\mathbf{f}_{11} - \mathbf{r}_{21})(\mathbf{\eta}_1 \mathbf{r}_{22} - \mathbf{\eta}_2 \mathbf{r}_{21}) \tag{9}$$

These can be determined, for example, by fitting the target vectors to a vector with all elements equal to c_{tot} . Eqs. (8) and (9) provide two relations between the elements of the matrix **R**, hence making two of them redundant.

In most cases, the spectra of some of the components can be determined in separate measurements. For example, the monomer–dimer equilibrium system can, in general, be diluted sufficiently to make the dimer concentration negligible. This enables the monomer spectrum to be record, which, of course, should be used as a constraint in the analysis. Normalizing the monomer spectrum to the same total concentration as the analyzed sample, one obtains from Eq. (4):

$$V_{\text{monomer}} = \eta_1 P_1' - \eta_2 P_2' = f_{21} P_1' - f_{22} P_2'$$
(10)

where $f_{21} = r_{11}$ and $f_{22} = r_{12}$ are determined by fitting the two projection vectors to the monomer spectrum. Equation (9) also provides two relations between the elements of matrix **R**. These are not independent of Eq. (7) and the two equations

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cannot be combined to solve for all the elements of matrix **R**, but they can be used to express **R** in a single element, below arbitrarily chosen to be r_{21} . Defined this way, matrix **R** produces **C** and **V** matrices that are consistent with the total sample concentration and the spectral response of the monomer. The value of r_{21} :

$$\mathbf{R} = \begin{bmatrix} f_{21} & f_{22} \\ r_{21} & 2f_{22} + (2f_{21} - r_{21})\frac{f_{11}}{f_{12}} \end{bmatrix}$$
(11)

determines the dimer spectrum and the monomer concentration profiles. Although all values of r_{21} produce mathematically acceptable solutions, reasonable results, in terms of spectral intensities and non-negative concentrations and spectral responses, are obtained over a relatively narrow range of r_{21} values. Still, the range is, in general, too large for a quantitative analysis.

The final constraint, which produces a unique solution, is the thermodynamic relation between temperature and the equilibrium constant. The concentrations of the components are related by the law of mass action:²⁰

$$K_{\rm D}(T) = \frac{c_{\rm X_2}(T)(c^0)^2}{c^0(c_{\rm X_2}(T))^2}$$
(12)

where $c^0 \equiv 1 \mod \text{dm}^{-3}$. Assuming that the dimerization constant $K_D(T)$ depends on the temperature according to the van't Hoff equation:²¹

$$\frac{\mathrm{dln}\,K_{\mathrm{D}}(T)}{\mathrm{d}(1/T)} = -\frac{\Delta H^{0}}{R} \tag{13}$$

where ΔH^0 is the molar enthalpy change, R = 8.31 J mol⁻¹ K⁻¹ is the gas constant and T is the temperature. r_{21} can now be determined by requiring that matrix **R** should rotate the target vectors to give concentration vectors (Eq. (3)) that produce an equilibrium constant whose logarithm is a linear function of 1/T. In practice, the solution is found by a simple search procedure. r_{21} is given an arbitrary value, for which a trial rotation matrix is calculated (Eq. (11)). This is used to calculate the trial concentration profiles (Eq. (3)), which are combined to the trial equilibrium constant (Eq. (12)). A linear regression of the equilibrium constants with respect to 1/T is then performed (Eq. (13)), which determines the trial enthalpy change of the reaction. Each trial rotation matrix also determines the trial spectral responses (Eq. (4)). The procedure is repeated for various values of r_{21} to find a range that produces reasonable concentration profiles and spectral responses. This is done rather arbitrarily since there is no simple way to estimate r_{21} . Once a range has been found, r_{21} is varied gradually in this range and a χ^2 (regression coefficient) is calculated for each regression of $\ln K_D(T)$ with respect to 1/T. The r_{21} that produces the best fit determines the matrix **R**. The analysis is readily performed with the Datan program.

EXPERIMENTAL

All the employed chemicals were of analytical reagent grade. The new cyanine dyes, were synthesized by Deligeorgiev *et al.*⁹ Stock solutions were prepared by dissolving a solid dye in buffer solution containing 500 mM KCl, 15 mM MgCl₂ and 100 mM tris(hydroxymethyl) amniomethane hydrochloride (Tris-HCl). The pH of all the solutions was kept constant at 7.50 using Tris buffer. The absorption spectra were recorded using an Agilent 8453 UV–Vis Diode-Array spectrophotometer, employing Agilent UV–Visible ChemStation software for data acquisition. A quartz cuvette of 1 mm optical path was used for all measurements. The pH measurements were made using a 300 HANA model pH-meter equipped with a combined glass electrode. Data preprocessing and data analysis were carried out in Matlab, version 7, and Datan computer programs.

RESULTS AND DISCUSSION

The absorption spectra of dyes 1–3 were recorded between 400 and 600 nm, in the temperature range 25–75 °C at 2.5 °C intervals and pH 7.50. As it was expected that the monomer form would be predominant over the dimer form with increasing temperature, the spectrum of the dye at the highest temperature was chosen as an initial estimate for the monomer in the subsequent calculations. Then according to Eqs. (1)–(13), the Datan program commenced with a trial value of r_{21} , at predefined interval, and iterated all the calculation steps. The iteration ceased when all r_{21} values in the initial interval had been tested. The K_D , the dimer spectrum and the ΔH corresponding to the minimum value of χ^2 were selected as the best values. χ^2 is a goodness of fit criterion and its value indicates the predicttability of the model, *i.e.*, how well the monomer spectrum and r_{21} were determined.

Two isosbestic points were found at 482 and 544 nm for dye 1 and at 498 and 550 nm for dyes 2 and 3. This suggests an equilibrium between two spectral species, *i.e.*, a monomer–dimer equilibrium. Accordingly, the total spectra were analyzed assuming them to contain contributions of the monomer and of the dimer. With increasing temperature the absorption peak for dyes 1–3 at around 520 nm grows and the absorption peak around 460 nm for dye 1 and around 490 nm for dye 2 and 3 diminished (Fig. 1). The dimerization constants, K_D , were calculated at different temperatures.

From the dependence of $\ln K_D$ on 1/T (Fig. 2), ΔH^0 and ΔS^0 values were determined. The dimerization constants at 25 °C and the thermodynamic parameters of the dimerization reactions of the dyes are given in Table I. As expected, K_D decreased with increasing temperature. The values of $\Delta H^0 < 0$ and $\Delta S^0 < 0$ for dimerization mean that the dimerization reaction is enthalpy-driven. It can be seen that the dimerization reactions of these dyes are exothermic and are characterized by relatively large negative ΔH values. The latter is consistent with dispersive van der Waals interactions of aromatic chromophores being the main contributors to complex formation.^{21,22} Dispersive van der Waals interactions are characterized both by negative enthalpy and negative entropy,²³ indicating an enthalpic origin for the dimerization processes of these dyes in aqueous solution. The relative

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dependence of the concentrations of the monomer and dimer of the dyes on temperature are shown diagrammatically in Fig. 3. The calculated absorption spectra of the monomer and dimer forms of the dyes are shown in Fig. 4.



TABLE I. Dimeric constant and values of thermodynamic parameters for dye 1 (3×10^{-4} M), dye 2 (1.9×10^{-4} M) and dye 3 (1.1×10^{-4} M) in water



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CONCLUSION

In this paper, for the first time, a thermodynamic study of the dimerization equilibrium of three new cyanine dyes in aqueous solution using the Datan package is reported. Dimerization constants, concentration profiles for the monomer and dimer, and spectral responses of monomer and dimer were obtained by computer refinement of temperature photometric titrations. The values of K_D decreased with increasing temperature. The thermodynamic parameters, enthalpy and entropy of dimerization reaction were calculated from the dependence of the dimeric constant on temperature (van't Hoff equation). The interaction between dye molecules is attributed predominantly to enthalpic rather than entropic reasons. The dimerization forces between the dye molecules are dispersive van der Waals interactions and K_D depends on the size and rigidity of the dye molecules.

извод

ТЕРМОДИНАМИЧКА КАРАКТЕРИЗАЦИЈА РАВНОТЕЖЕ ПРОЦЕСА ДИМЕРИЗАЦИЈЕ НОВОСИНТЕТИСАНИХ ПОЛИМЕТИН-ЦИЈАНИНСКИХ БОЈА

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Равнотежа мономер–димер и термодинамика три нове цијанинске боје ипитиване су спектрофотометриском и хемометријском методом. Константе равнотеже процеса димеризације ових нових цијанинских боја одређене су на основу зависности њихових абсорпционих спектара од температуре у опсегу од 25 до 80 °C при концентрацијама од $3,0 \times 10^{-4}$, $1,9 \times 10^{-4}$ and $1,1 \times 10^{-4}$ M за боју **1**, **2**, и **3**, респективно. Обрада података, у смислу квантитативне анализе спектара, заснована је на симултаној анализи преклопљених абсорпционих трака у комплетном опсегу таласних дужина. На основу температурне зависности константе равнотеже за процес димеризације одређене су промена енталпије и ентропије овог процеса.

(Примљено 14. септембра 2007, ревидирано 17. јуна 2008)

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