BIOASSAY OF PURINE ALKALOIDS ON THIN LAYER CHROMATOGRAPHY AGAINST MOULDS

Patrycja W. Kwaśniewska[a], Anna K. Przybył[b], Dariusz Kikut-Ligaj[c], Grzegorz Cofta[a]

[a] Poznań University of Life Sciences, ul. Wojska Polskiego 38/42, 60-637 Poznań, Poland , [b] Adam Mickiewicz University in Poznań, ul. Umultowska 89b, 61-614 Poznań, Poland, [c] Poznań University of Economics Al. Niepodległości 10, 61-875 Poznań, Poland

ABSTRACT

The main purpose of the introduced project is to find a model compound describing steric and electronic properties which are necessary to ensure optimal supramolecular interactions with a specific proteins - microfungi. In this test the model bases on selected methylxanthine alkaloids contain three nucleophilic active sites B_1, B_2 and B_3: C_2=O; C_6=O and on the tertiary nitrogen atom N_9.

In this study the properties against microfungi were analyzed using the microbiological test of TLC-bioautography method in determination of effective alkaloid concentrations for 100% growth reduction (LD_{100}).[1]
The microfungal strains used in the bioassay were *Aspergillus niger* van Tieghem and *Penicillium cyclopium* Westing. All purine derivatives used in the microbiological screening test: xantine (**1**), theobromine (**2**), theophylline (**3**), aminophylline (**4**), caffeine (**5**), 7-(β-hydroxyethyl)theophylline (**6**), 7-(2-hydroxypropyl)teophylline (**7**), 7-(2,3-dihydroxypropyl)theophylline (**8**), theophylline-7-acetic acid (**9**) showed inhibitory effect against moulds.

INTRODUCTION

Methylxanthine is a particular group of purine alkaloids. It consists of three main members:
caffeine, theophylline and theobromine shared by their xanthine skeleton. This group of alkaloids are compounds of plant origin (coffee, tea, guarana and yerba mate) which are generally found in food products at various degrees of processing. Plants use them as a natural pesticide against insects.[2] Methylxanthine has many antimicrobial effects on different types of organisms and they are also components of pharmaceuticals and nutraceuticals.

Investigation of antimicrofungi analyzed using a microbiological assay and used 3D pharmacophore model creation to explain how methyloxantines binds to the receptor protein a series of structurally similar methylxanthine alkaloids were analyzed. Pharmacophore is a model describing the spatial relationships between the elements common to ligands interacting with receptor (specific biological target structure).[3] The process of creating a simple pharmacophore for methylxanthine alkaloids is presented in the figure below (Fig.1).

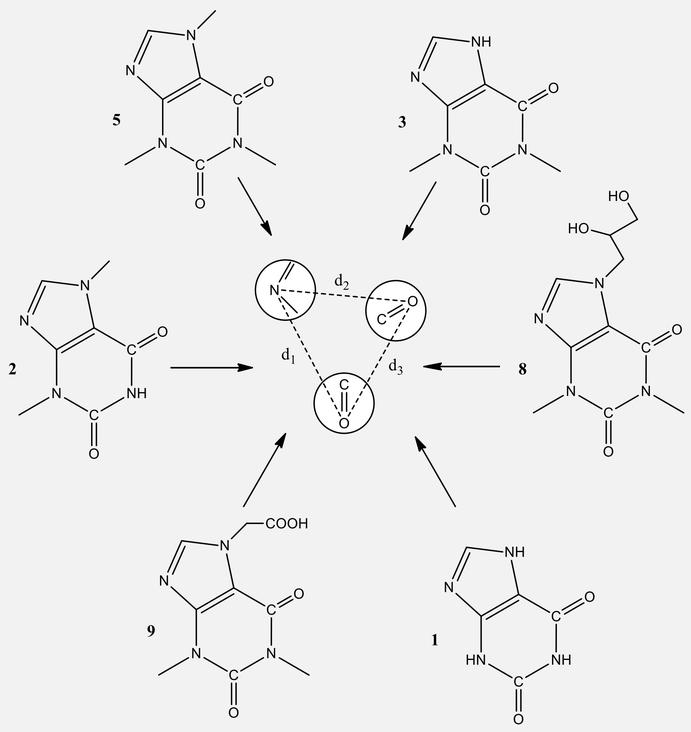

Fig.1 Three-point pharmacophore model

3D PHARMACOPHORE MODEL CREATION

Progress in modern methods of molecular modelling has permitted more accurate insight into the conformation of biologically active compounds and enabled undertaking attempts at finding correlation between microfungi and the structure of these compounds. Due to their distinct spatial structure, we have taken an interest in the group of purine alkaloids.

The computer calculations were performed in the Gaussian 09W application. The geometry of caffeine and the other methylxanthine alkaloids was optimized by means of DFT using the B3LYP functional with the 6-31G(2d,p) basis set.

The pharmacophore model applied for the analysis contains three key areas in the recognition of the studied methylxanthine alkaloids by a specific receptor protein - microfungi. The three-point pharmacophore model contains nucleophilic active sites located on carbonyl groups (C_2=O, C_6=O) and on the tertiary nitrogen atom N_9.

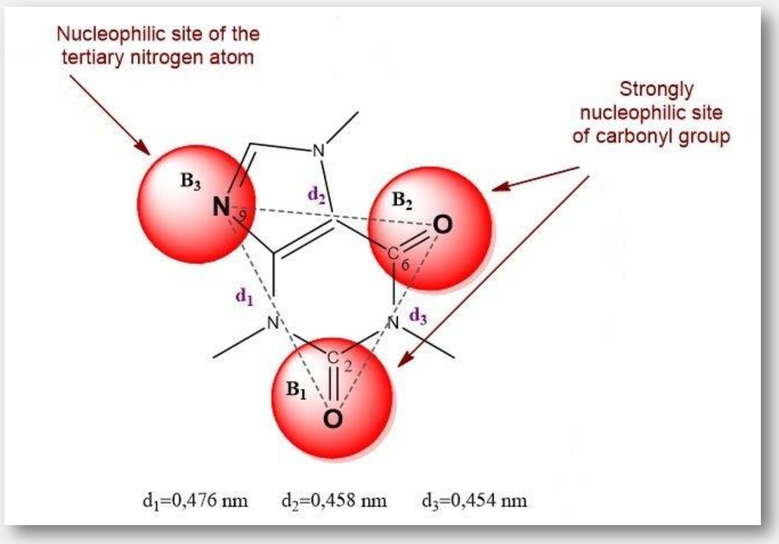

Fig.2 Map illustrating the spatial arrangement of nucleophilic active sites

R_1	R_2	R_3	R_4	comp.
H	H	H	-	**1**
CH_3	H	CH_3	-	**2**
CH_3	CH_3	H	-	**3**
CH_3	CH_3	-	H	**4**
CH_3	CH_3	CH_3	-	**5**
CH_3	CH_3	C_2H_4OH	-	**6**
CH_3	CH_3	C_3H_6OH	-	**7**
CH_3	CH_3	$C_3H_5(OH)_2$	-	**8**
CH_3	CH_3	CH_2COOH	-	**9**

ANTIFUNGAL ASSAY

Bioautography belongs to microbiological screening methods generally used for the detection of antifungal activity. The procedure in bioautographic methods is used in agar diffusion methods. In this study the properties against microfungi were determined by using TLC-bioautography method. The difference is that the tested compounds of methylxanthine alkaloids diffuse to inoculated agar medium from the chromatographic layer which is silica gel support (TLC).
This method is used to quickly verification of antifungal properties of practically every compound. The results allowed to determine the leading compounds which the derivatives can be a potential component of a new fungicide.

Microfungi like e.g. moulds can cause considerable economical losses and they can also have harmful effect on human health. An interesting way of approaching this problem is search of biocide from natural products.
In our study 4µm of each of the purine derivatives (purchased from SIGMA) were placed on the silica gel support (TLC) in determination of effective alkaloid concentrations for 100% growth reduction (LD_{100}). Most of them were soluble in water. TLC plate are placed on the inoculated agar surface in sterile Petri dishes. Agar medium were prepared with Czapeks Dox, agar and malt extract. Each experiment was repeated three times with triplicates of each compounds. The fungistatic activity of the alkaloids tested was compared with commercial fungicides IPBC (3-iodo-2-propynyl butylcarbamate). Plates were incubated at 28°C for 1 week and the air humidity was 95%. The microfungal strains used in the bioassay were *Aspergillus niger* van Tieghem and *Penicillium cyclopium* Westing.

RESULTS AND DISCUSSION

Bioautography combined with TLC (TLC-bioautography) showed that the growth of all fungal strains was inhibited by all purine derivatives forming visible inhibition zones. The results of bioassay tests are shown in Table 1.

The microfungi on the second day of the study infected the entire surface of the medium and they showed good growth in conditions without the presence of the compound. The highest effectiveness in relation to all test microfungi: A. *niger* and *P. cyclopium* was exhibited by 7-(2,3-dihydroxypropyl)theophylline.
After five days was observed in the data alkaloids fungal sporulation of test but the effect was beginning to lessen in compared to the reference test. It is interesting that the mixture of 7-(2,3-dihydroxypropyl)theophylline and theobromine showed higher antifungal activity than 7-(2,3-dihydroxypropyl)theophylline alone. It indicates that a synergistic effect by both alkaloids may have occurred in the antifungal activity. Used good conditions of the test and the results can be considered reliable.

comp.	24h	48h	72h	96h	120h
1	0	1	1	2	2
2	0	1	1	2	2
3	0	1	1	2	2
4	0	1	1	2	2
5	0	1	1	2	2
6	0	1	1	2	2
7	0	1	1	2	2
8	0	0	1	1	2
9	0	1	1	2	2
Ref.	1	2	2	2	2

Table 1. 0 – no growth, 1 - growth of hyphae without spores, 2 - sporulation mycelium

REFERENCES:

1.I. M. Choma, E.M. Grzelak, J. Chromatogr. A (2011), 1218, 2684-2691.
2.A. Nehlig, J-L. Daval, G. Debry, Brain Research Reviews (1992), 17, 139-170.
3.A. Bielenica, J. Kossakowski, *Zastosowanie metod obliczeniowych do wyznaczania budowy modeli farmakoforowych receptorów 5-ht$_{1a}$, 5-ht$_{2a}$ oraz 5-ht$_{7}$,* Biul. Wydz. Farm. WUM (2010) 1, 1-12

ACKNOWLEDGEMENTS:

The study was supported by Norway Grants and the National Centre for Research and Development of Poland (NCBiR) as a part of Polish–Norwegian Research Programme: *Superior bio-friendly systems for enhanced wood durability.* No. Pol-Nor/203119/32; DURAWOOD).