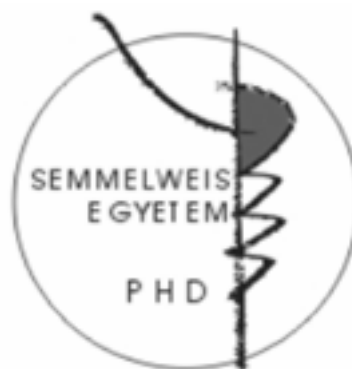


# Synthesis, physicochemical and pharmacological properties of pentacyclic alkaloid-analogues

Ph.D. Theses

**Máté Dániel Bubenják**

Semmelweis University  
Doctoral School of Pharmaceutical and Pharmacological Sciences



Supervisor: Dr. Béla Noszál D.Sc.

Opponents: Dr. Cecília Pápay-Sár Ph.D.  
Dr. Andrea Czompa Ph.D.

Chair of exam committee: Dr. György Bagdy D.Sc.  
Exam committee: Dr. Valéria Kecskeméti D.Sc.  
Dr. Pál Perjési D.Sc.

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## Summary

Quinazolinocarboline rutaecarpine and evodiamine (*Evodia rutaecarpa*) are main alkaloid components of traditional Chinese folk-remedies. Several new valuable activity of these pentacyclic alkaloids have been discovered by pharmacological researches in the 1990s. Evodiamine exhibited selective antitumor and antimetastatic effects on several cancer cell lines and became lead structure of anticancer agents.

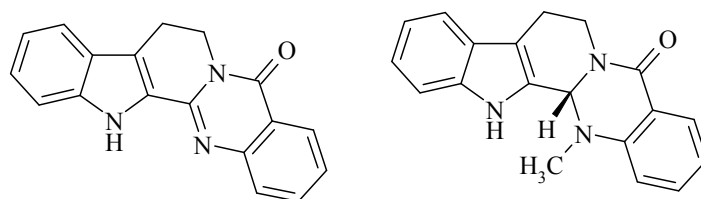
During our synthetic research we achieved to gain alkaloid hybrid derivatives by combining the structural elements of quinazolinocarbolines with analogous alkaloids or drug molecules having similar effects by bioisosteric replacements. 8-norrutaecarpine, a hybrid molecule of rutaecarpine and luotonin A containing the indolo-pyrroloquinazolinone ring system has been synthesized. The hybrids of rutaecarpine and piroxicam bearing the indolo-pyridobenzothiazine and the 12-azaindolo-pyridobenzothiazine structures were prepared on two alternative routes. Two new heterocondensed pentacyclic compounds, 5-sulfarutaecarpine and 5-sulfa-8-norrutaecarpine were reached via bioisosteric replacement on the structure of rutaecarpine and 8-norrutaecarpine. Two new tricyclic ring systems, pyrido-benzothiadiazine and pyrrolo-benzothiadiazine were produced as intermediaries of these pentacyclic molecules. Series of substituted derivatives were prepared for pharmacological studies by modification of the structures with various substituents and solubilizing groups. During our work alternative way for synthesis of nauclefine (*Nauclea latifolia*) was laboured, and we published the synthesis of indolilquinazolinone derivative bouchardatine (*Bouchardata neurococca*) for the first time.

Some of the physicochemical attributes of the synthesized intermediaries were defined, such as the pKa constants of 2,3-polymethylene-benzothiadiazines. Proton/deuteron exchange kinetic constants of active methylene-groups of five tricyclic compounds were measured by <sup>1</sup>H NMR technique. Solvent-dependent ratio of the *Z/E* isomers of phenylhydrazone-derivatives in polar and apolar solvents were determined.

In the case of 18 produced compounds our work was completed by *in vitro* pharmacological studies performed within co-operation with the *Institute of Pharmacology*. The viability of HeLa cells was inhibited by five of our compounds to similar extent as the effect of evodiamine. Eight of our compounds induced apoptosis on HeLa cells to similar extent as evodiamine.

## Introduction

During the elaboration of my dissertation I was able to join the series of synthetic researches running in co-operation between the *Semmelweis University Department of Pharmaceutical Chemistry* and the *Chinoin Pharmaceutical and Chemical Works Ltd.* In the course of these researches in the study of nitrogen-bridgehead compounds development of several original methods and patents were realized, original ways of synthesis of quinazolinocarboline alkaloids, such as rutaecarpine and its derivatives were successfully found, which are the easiest and the most economic totalsyntheses upto this day according to the qualification of Merck Index. The quinazolinocarboline heterocycle alkaloids, rutaecarpine and evodiamine are main alkaloid components of the traditional folk medicine, that have been used in the Eastern medicine for thousands of years. The dried fruit of *Evodia rutaecarpa* (“Wu-Chu-Yu”) is one of the 200 most widely applied Chinese pharmaceutical products, the main agents whereof are rutaecarpine, evodiamine and of their substituted derivatives.



**Figure 1. Structure of rutaecarpine and (S)-evodiamine.**

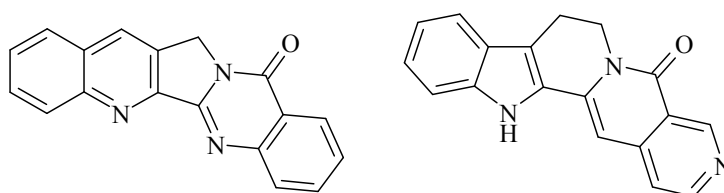
Several procedures were worked out for synthetic production of pentacyclic ring systems from the total synthesis of Nobel prize winner *Robinson* to the synthetic approach of *Jan Bergman*, the professor of chemistry of the Swedish *Karolinska Institute*. Pharmacological characterization of quinazolinocarbolines by the study methods of the modern science had been developed only since the 1990s. The studies proved the healing effects of natural plant extracts and discoveries were made through which these molecules became lead structures of novel medicine developments.

Rutaecarpine possesses a remarkable antihypertensive effect, as it activates vanilloid receptor-1 throughout stimulation of CGRP release. An anti-inflammatory effect of rutaecarpine was also found, owing to its selective and extremely intense COX-2 isoenzyme-inhibiting effect.

Japanese researchers found during the migration-studies of the metastasis-inducing property of cancer cells that evodiamine possesses a unique, non-cytotoxic antimetastatic effect selectively to cancer cells. After 2001 pharmacological research of evodiamine has been widely expanded in this direction, the compound has been tested on several cell lines with positive results.

## Objectives

Chemical reactivity studies of total synthesis intermediaries of rutaecarpine-type alkaloids were planned based on the observations and achievements gleaned in the field of heterocyclic chemistry in the *Department of Pharmaceutical Chemistry*. Production of similar ring systems, isosteric and bioisosteric derivatives fitted in our plans. During our work novel reaction routes for building up of original, newly described ring systems were planned. By means of these reaction routes, we managed to develop more selective, more effective heterocyclic variants and derivatives with better solubility of the multiple biological effect-possessing rutaecarpine, evodiamine, luotonin A and nauclefine. These basic structures and derivatives possess better bioavailability than the compounds of natural origin and show the selective antitumour pharmacological characteristics of evodiamine. We intended to discover such alternative synthetic routes to this group of natural alkaloids, that are more economic, provide novel production methods that afford new substitution opportunities, and proceed from substances that are easily available and at low cost.



**Figure 2. Structure of luotonin A and nauclefine.**

We wanted to discover and characterize the remarkable physicochemical characteristics of these novel structures produced by our synthetic work.

Furthermore, our aim was to complete our work with pharmacological studies, thus verifying the antitumour and apoptosis-inducing effect of the produced compounds. We proposed to perform measurements of changes in viability of HeLa cells, to measure induced apoptosis and to monitor changes in caspase-3 enzyme activity.

## Methods

### Structure-analysis methods used for identification of the synthesized products

Reaction progression during the organic syntheses was monitored by thin layer chromatography (TLC) tests of samples taken directly from the reaction compound in appropriate intervals. Benzene:methanol 4:1 on silica gel plates (Merck) was used as eluent, Dragendorff-reagent and ninhydrine were used for visualization. The melting points of the intermediaries and the products were measured by Stuart Scientific SMP apparatus; UV-spectra were recorded by

UNICAM SP-800 and Jasco V-550 spectrophotometer. IR spectra were recorded by PYE UNICAM SP-1100 IR-spectrophotometer from KBr.

The NMR-spectra of the compounds were measured by Varian Unity Inova 400, 500 and 600 MHz (Palo Alto, CA) apparatuses in DMSO-*d*<sub>6</sub>, in D<sub>2</sub>O, and in some cases in CDCl<sub>3</sub>. Where it was necessary, we detected <sup>13</sup>C spectra as well besides <sup>1</sup>H spectra, and in some cases 1D NOESY and two- and three-dimensional homo- and heteronuclear techniques were applied (2D COSY, HSQC és HMBC). Varian VnmrJ and Mestre-C 4.8.6.0 (Mestrelab Research, Santiago de Compostela, Spain) softwares were used for the evaluation of measurement results.

<sup>1</sup>H NMR-pH titrations were conducted in a probe tube at 2 mmol/dm<sup>3</sup> concentration at 25 °C, 0.15 mol/dm<sup>3</sup> ionic strength (NaCl) in H<sub>2</sub>O:D<sub>2</sub>O 9:1 solution. *In situ* pH monitoring was accomplished by using trimethylamine, TRIS, imidazole, acetic acid, chloroacetic acid and dichloroacetic acid (c= 1 mmol/dm<sup>3</sup>).

Mass spectra were recorded by an Agilent 6410B triple quadrupole apparatus with electrospray ionization (ESI), results were estimated by the Masshunter B.01.03 software. Element analysis (C, H, N) was conducted by the Perkin Elmer 2400 CHN analyser of the Chinoin Pharmaceutical and Chemical Works Ltd.; discrepancy to the calculated values was within the following ranges: C: ± 0,30; H: ± 0,21; N: ± 0,26%.

## **Methods, substances and devices of pharmacological studies**

### ***Assessment of viability of HeLa cervical cancer cells to study pro-apoptotic effect***

On a 96-chamber plate 5000 HeLa cells per chamber were treated with our substances solved in the compound of the medium (99%) – DMSO (1%) for 48 hours. At the end of the incubation period cells were treated with a tetrazolium salt (XTT), then the number of cells survived were assessed with an ELISA reader. The results were compared with the blank (treated with appropriately diluted DMSO) experiment thus we were able to conclude to the change in the proliferation of the cells.

#### *Experiment protocol:*

##### *1. Solutions:*

- Medium: DMEM + 10 % FBS + 2 mmol/dm<sup>3</sup> L-glutamine + gentamycine.
- XTT: 0,9 mg/ml, newly solved in the medium (by gentle warming).
- PMS (phenazine methosulfate): 3 mmol/dm<sup>3</sup> solution (dissolved in H<sub>2</sub>O), incubated at -20°C.
- Compounds: c= 10<sup>-2</sup>, 10<sup>-3</sup> and 10<sup>-4</sup> mol/dm<sup>3</sup> (dissolved in DMSO), incubated at -20°C.

*2. Treatment:* 5000 HeLa cells/chamber, transparent, 96 chamber plate. Five calibration points were taken within the range of 10000-625 cell number with 2:1 dilution, to assess the linearity

of cellnumber/absorbance coherency. Treatment was conducted the day after having the cells placed on plate, in  $10^{-4}$ ,  $10^{-5}$  and  $10^{-6}$  mol/dm<sup>3</sup> concentration of the compound throughout 48 hours. Main solutions of the compounds were diluted to 1:100 in volume by media before usage, then cells were treated by the thus gained solution in 200  $\mu$ L.

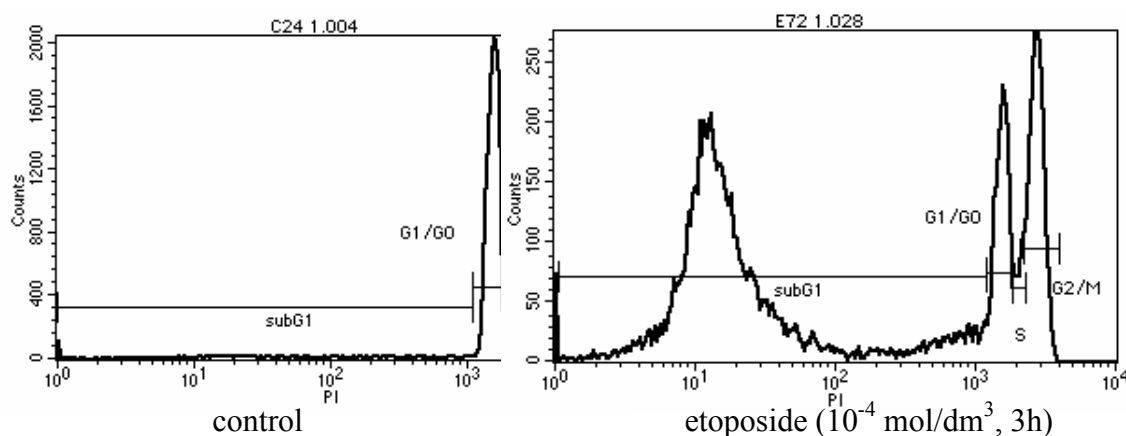
3. *Processing, measurement*: The viability of cells after the treatment period was assessed by the XTT reduction method. The medium was replaced by 100  $\mu$ L new medium, then 50  $\mu$ L XTT solution was added to (solved in 0,9 mg/ml XTT medium + PMS diluted to 1:50). Measurement on ELISA reader at 450 nm, background measurement: at 620 nm, every half an hour.

### ***Detecting of nucleosomic DNA-fragmentation with flow cytometry***

With the help of flow cytometric measurement we are able to assess the ratio of cells containing fragmented DNA in the samples. Since DNA-fragmentation is merely characteristic to cells perished in apoptosis, we are able to assess the extent of apoptosis induced during the treatment in the cell culture. Besides evodiamin and rutaecarpine, etoposide was used as positive control as well.

#### *Treatment protocol:*

1. *Solutions*: a.) Medium: DMEM + 10 % FBS + 2 mmol/dm<sup>3</sup> L-glutamine + gentamycine.  
b.) Compounds:  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$  mol/dm<sup>3</sup> concentration solved in DMSO, incubation -20°C.
2. *Treatment*: 100.000 HeLa cells/sample, treatment with  $10^{-4}$ ,  $10^{-5}$  and  $10^{-6}$  mol/dm<sup>3</sup> concentration of the tested compound for 72 hours.
3. *Fixation in ethanol*:  $2 \times 10^5$  cells were fixed in 1 ml -20°C 70 % ethanol, 30 minutes incubation at room temperature.
4. *Dying*: Cells were centrifuged, 1 ml of extraction buffer (200 mmol/dm<sup>3</sup> Na<sub>2</sub>HPO<sub>4</sub>, pH set to 7,8 with 200 mmol/dm<sup>3</sup> citric acid + 0,1 mg/ml RNase A) was poured on the sediment. After 15 minutes of incubation at room temperature, 10  $\mu$ g propidium iodide was added to it, followed by 15 minutes of incubation.



**Figure 3. Diagram provided by flow cytometry. G2/M: diploid cells, G1/G0: haploid cells, subG1: apoptotic cells.**

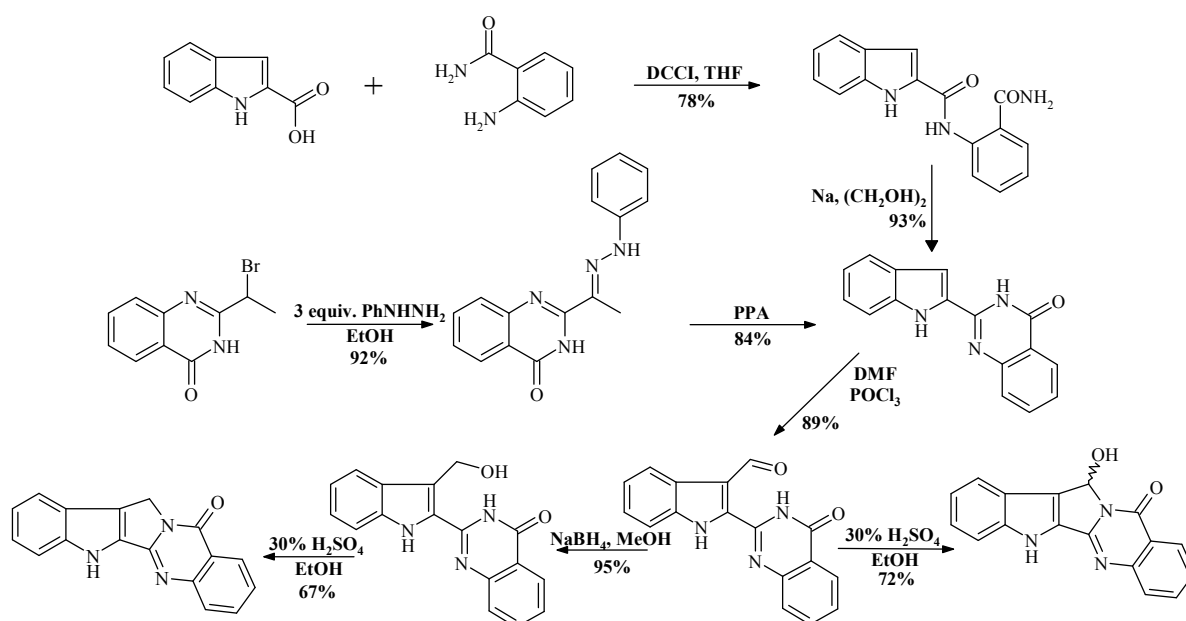
5. *Measurement*: Data of FL2 fluorescence channel registered by flow cytometry (on logarithmic and linear scale as well). Gating was set on the one hand on the cell scale (FSC) – FL2 log scale diagram (exclusion of debris), on the other hand on the linear signal range – signal-width diagram (exclusion of clotted cells).

### *Measurement of caspase-3 enzyme activity*

Isolated procaspase-3 enzyme was treated with the solution of our compounds in  $10^{-6}$  mol/dm<sup>3</sup> concentration for 24 hours, then a synthetic caspase-3 substrate, Ac-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC) was added to the system. Fluorescent 7-amido-4-methylcoumarin (AMC) was released from the substrate, that was detected with fluorescence spectroscopy. Its amount was equivalent to the amount of the activated caspase-3 enzyme. Other conditions of the measurement were set according to the literature we referred to.

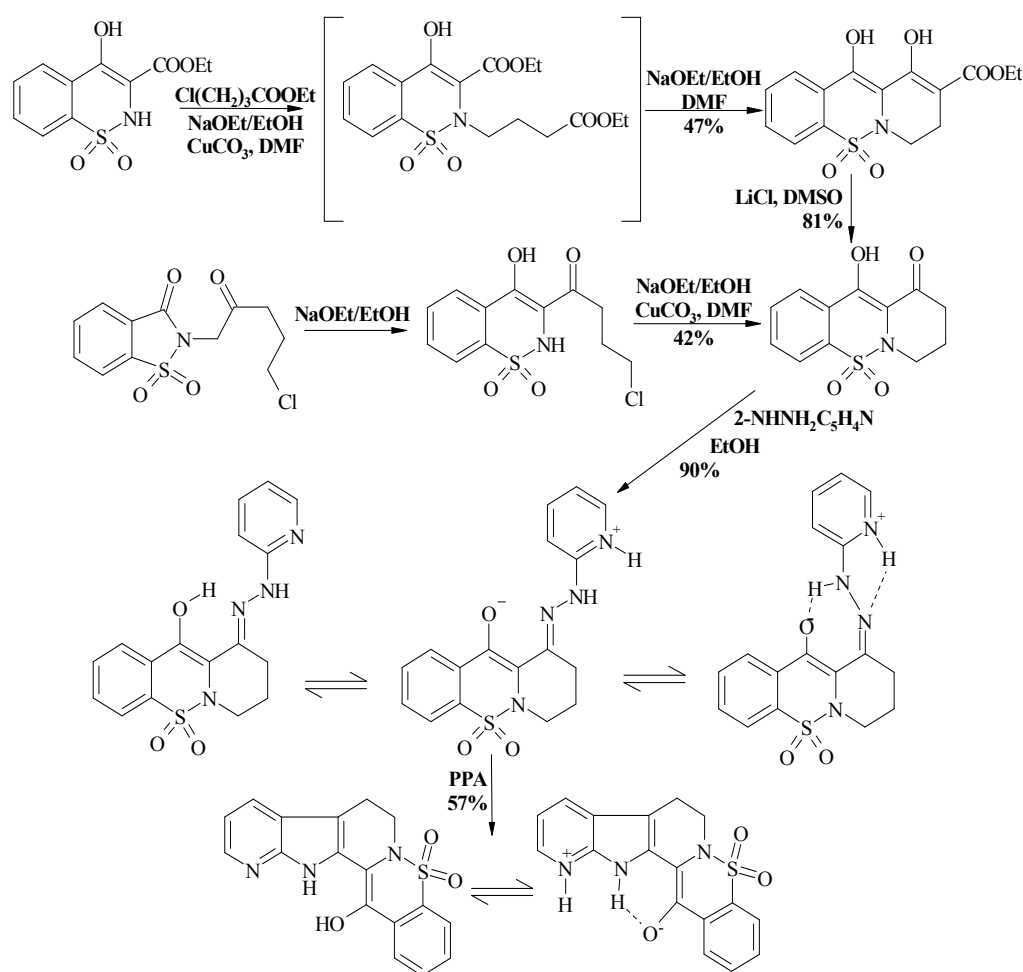
## Results and conclusions

During our researches we worked out new and modified methods for production of quinazolinocarboline alkaloid derivatives with ring homologue and substituted hetero atoms. By performing bioisosteric replacements in the ring system (aza-analogues, and amide-sulfonamide substitution), by producing nor-, and seco-derivatives, and by working out of reaction pathways able to input solubilizing groups we got the opportunity to develop pharmacons with better pharmacokinetic profile than the natural alkaloids, containing new heterocyclic ring systems, and we were able to study their structure-activity relationships more comprehensively. New derivative composing routes for focused development of active molecules were discovered and processed.



**Figure 4.** Synthesis of 8-norrutaecarpine and 7-hidroxi-8-norrutaecarpine via bouchardatine.

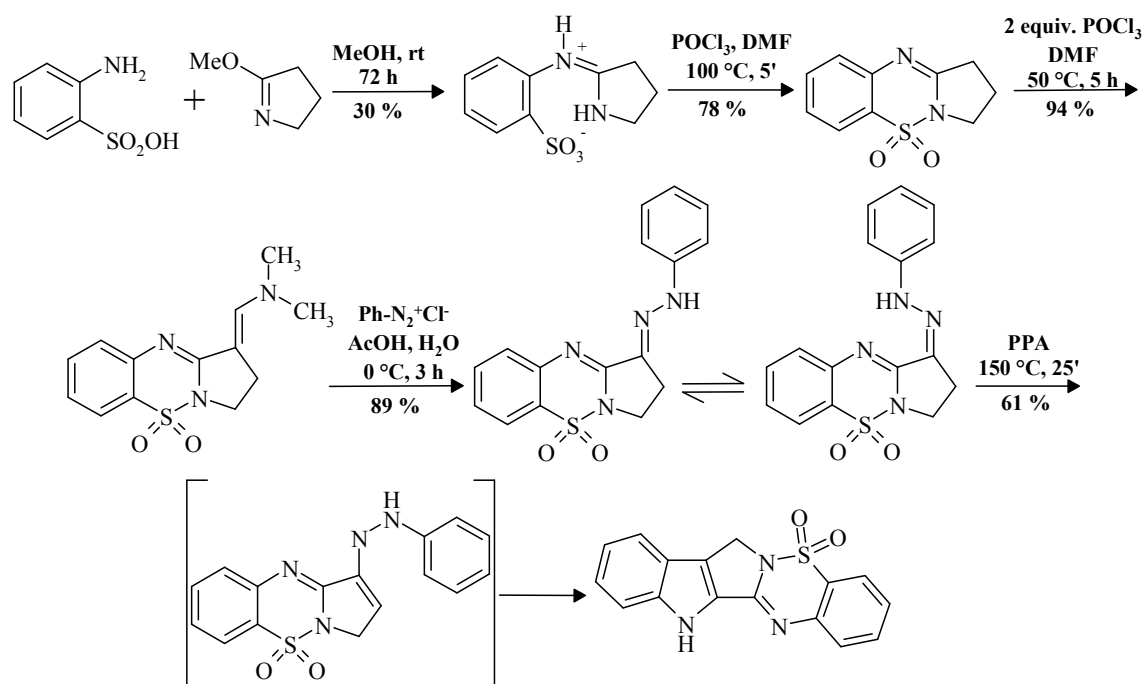
We managed to produce 8-norrutaecarpine in two alternative ways. By condensation of indol-2-carboxylic acid and anthranilamide indolil-quinazolinone was obtained. This compound was produced as well when we treated 2-bromine-ethyl-quinazolinone with phenylhydrazine, and the Fischer-indole synthesis in polyphosphoric acid of the phenylhydrazone derivative yielded from the nucleophilic substitution followed by an autooxidation. The 3-formyl derivative was reached selectively by the Vilsmeier-Haack formylation of indolil-quinazolinone. From the 3-formyl derivative we reached 8-norrutaecarpine via reduction and acidic ring-closure. Via direct ring-closure 6-hydroxy-8-norrutaecarpine (14-norluotonin B) was gained.



**Figure 5. Synthesis of hybrid structure of rutaecarpine and piroxicam.**

Benzothiazines and benzothiadiazines possessing anti-inflammatory effect are basic structures of several medicines, and through the COX-1/2 inhibitory effect on prostaglandine PG-E2 and PG-E4 they block the migration of cancer cells and formation of metastases. Deliberating the bioisosteric characteristics of the structural elements such as quinazolinone, benzothiazine and benzothiadiazine, we planned such new heterocyclic derivatives, in which the quinazolinone units in the ring system of pentacyclic alkaloids were replaced by the isosteric structures mentioned above.



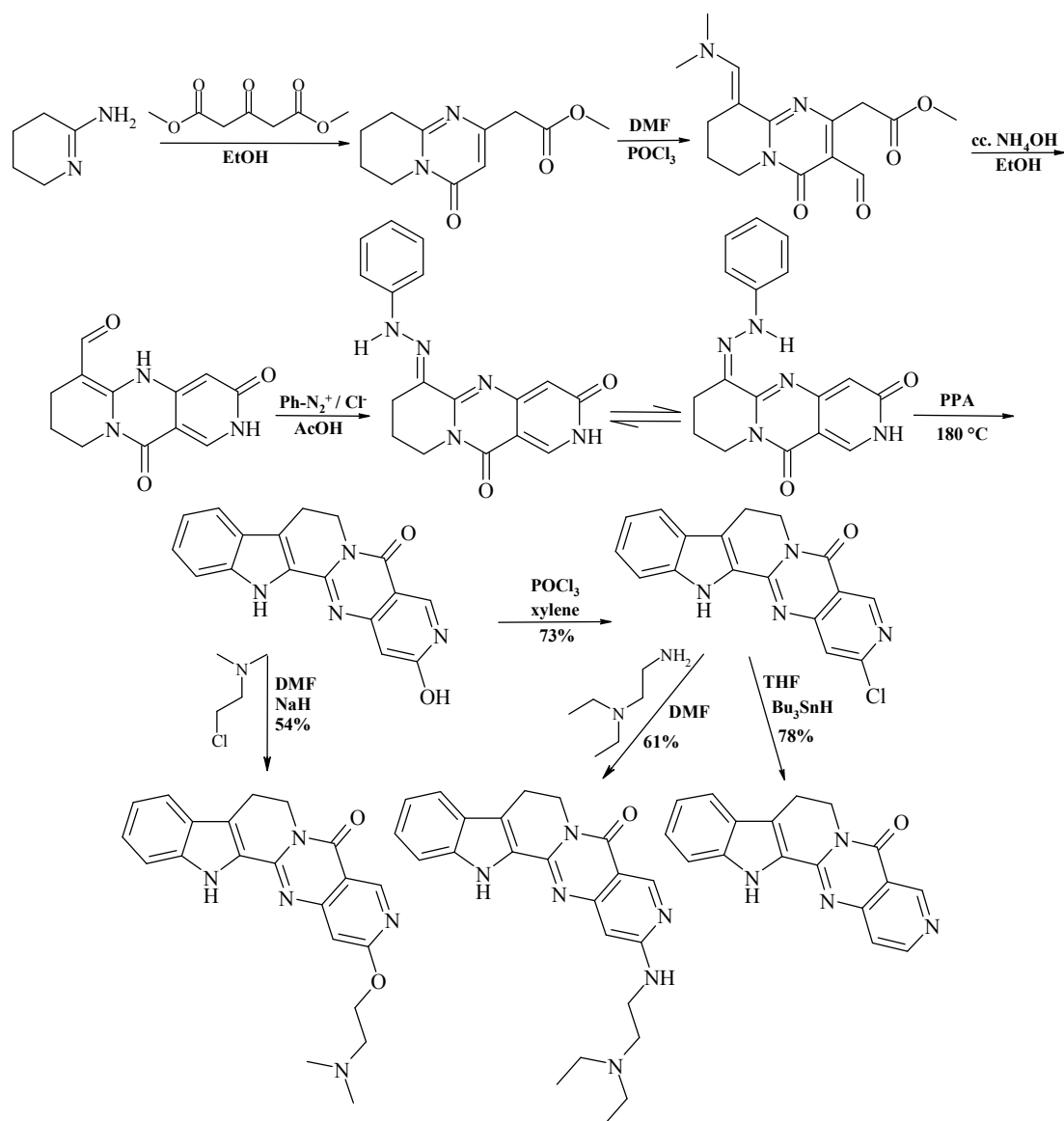


**Figure 6. Synthesis of 5-sulfa-8-norrutaecarpine via pyrrolo-benzothiadiazine.**

Ortanilic acid condensed with 2-methoxy-3,4-dihydro-5H-pyrrol, the gained sulfonic acid derivative was cyclized with phosphoryl chloride. The active methylene-group reactivity of pyrrolobenzothiadiazine is not sufficient for the realization of direct azo-coupling, therefore we wanted to develop a formyl group as activating substituent through the Vilsmeier-Haack formylation. The yielded product was the dimethylaminomethylene derivative which possesses great stability. The reaction of the compound with diazonium salts lead to the desired phenylhydrazone derivatives with sufficient yield and the synchronized elimination of the activating group happened as well. Fischer-indole synthesis started from phenylhydrazone yielded 8-nor-5-sulfarutaecarpine.

A new tricyclic ring system was developed by following the course of piroxicam synthesis started from saccharine, that was condensed with arylhydrazines. Interesting geometrical isomer equilibriums were identified among the different protonated forms of the yielded arylhydrazones. The indole synthesis of the 2-pyridilhydrazone derivative yielded only under extreme conditions and with moderate yield the pentacyclic ring system of the bioisosteric hybrid of piroxicam and rutaecarpine.

Nauclefine, the alkaloid having indoloquinolizidine structure was isolated in 1975 from the husked cortex of the root of african peach (*Nauclea latifolia*), to which Bergman has already drawn attention in his summary in 1983. Rutaecarpine varies in the position of one N-atom from the anti-leukaemic nauclefine. Therefore he supposed rightly the expectable similar characteristics of rutaecarpine derivatives.

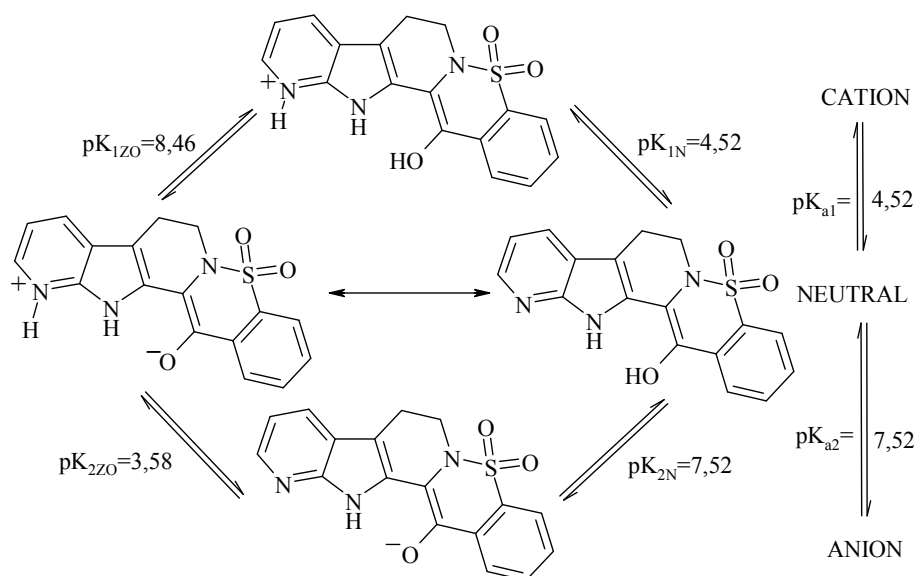


**Figure 7. Synthesis of 2-hydroxy-3-azarutaecarpine and its substituted derivatives.**

The synthesis of the basic skeleton of the two alkaloid hybrids was published earlier by our research group. During our recent work a novel total synthesis was worked out in order to produce different substituted derivatives with the utilization of pyrido-pyrimidines. 2-Hydroxy-3-aza-rutaecarpine, important in developing derivatives substituted on the E-ring, was reached by azo-coupling and indole synthesis of the tricyclic compound yielded by formylation of 4-oxo-pyrido-pyrimidine-2-acetic ester and by cyclization with ammonia. A tertiary amine, that improves the solubility via aliphatic linkers and enables salt formation, was attached to the pentacyclic ring system.

#### ***pKa prediction of our compound by an Australian research group***

A macroscopic and microscopic pKa-predicting method based on quantumchemical calculations was developed for the structures of anti-inflammatory oxicams by Australian and Mexican researchers, which was validated on experimental data of five oxicam derivatives. They performed calculations to assess the pKa values of the pentacyclic compound firstly syn-

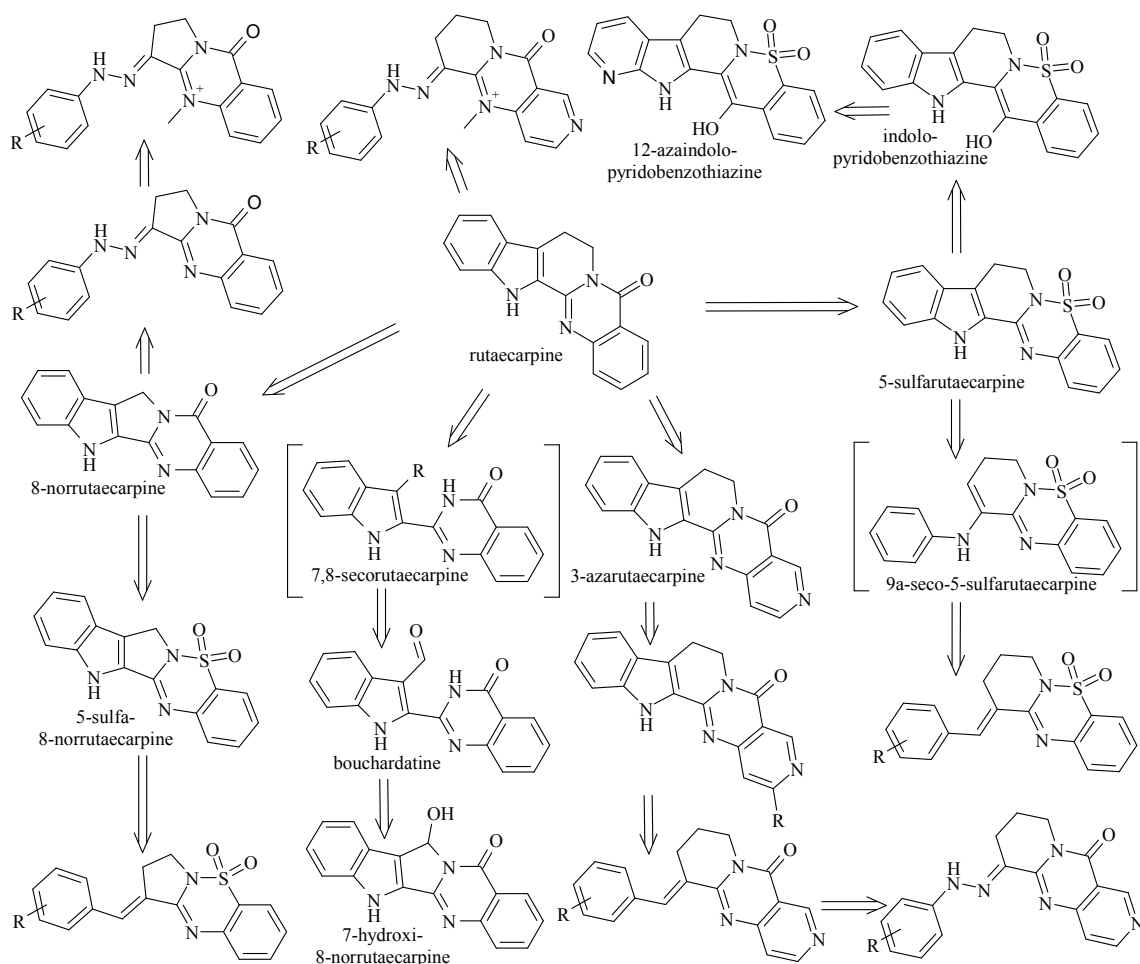


**Figure 8. Scheme of microspecies and predicted microscopic and macroscopic protonation constants of 14-hydroxi-12-azaindolopyrido-1,2-benzothiazine 5,5-dioxide.**

thesized by us, 14-hydroxi-12-azaindolopyrido-1,2-benzothiazin 5,5-dioxide. The published calculated microscopic and macroscopic pKa constants are shown on Figure 8. The main protonation pathway based on the data is the enolate-enole then piridine-piridinium route, which goes throughout the neutral microspecies, so the importance of zwitterionic species is negligible. This pathway also seems more preferable according to the main chemical properties of the proton-acceptor groups.

### Summary

- Two heterocondensed tricycles (tetrahydropyrido-benzothiadiazine, dihydropyrrolo-benzothiadiazine), unknown thus far in the literature, were produced via amide-sulfonamide bioisosteric replacement of mackinazolinone and deoxyvazicinone.
- Five new pentacyclic compounds were deduced from alkaloid structures via bioisosteric replacements, their syntheses were accomplished and several of their substituted derivatives were produced (5-sulfarutaecarpine, 5-sulfa-8-norrutaecarpine, 8-norrutaecarpine, 7-hydroxi-8-norrutaecarpine, indolo-pyridobenzothiazine, 12-azaindolo-pyridobenzothiazine).
- Alternative route for synthesis of a familiar pentacycle and for production of their substituted derivatives were developed (3-azarutaecarpine and its E-ring substituted derivatives).
- The total synthesis of a natural alkaloid, bouchardatine was accomplished for the first time by us – in two alternative ways.
- A series of 41 compounds was created from derivatives of three tricycles condensed with substituted arylaldehydes.



**Figure 9. Biososteric derivation of the produced compounds from rutaecarpine.**  
Hydrazones, aldehydecondensed tricycles, pentacycles and the bouchardatine.

- A 62-member series of substituted hydrazone derivatives were created from four tricyclic compounds.
- pKa values of 2,3-polymethylene-benzothiadiazines were assessed.
- Kinetic constants of solvent-dependent conformational alteration of bicyclic precursors of 2,3-polymethylene-benzothiadiazines were assessed.
- Proton/deuteron exchange kinetic constants of the active methylene groups of five tricyclic compounds were measured with  $^1\text{H}$  NMR.
- Solvent-dependent ratio of *Z/E* isomers of seven hydrazone derivatives was assessed in polar and apolar solvents.
- During our pharmacological examinations the viability of cancer cells treated with the 18 compounds produced by us, the apoptosis inducing effect of our compounds on HeLa cervix cancer cell line, and their effects affecting the functioning of caspase-3 enzyme *in vitro* were studied. The viability of HeLa cells was inhibited to similar extent as evodiamin by five of our compounds, while eight of our compounds induced apoptosis on the HeLa cell line to the same extent. Evodiamine exerts its anti-cancer activity via various mechanisms (caspase-activation, NF- $\kappa$ B-regulation, MAPK-inhibition, affecting bcl/bax equilibrium, an-

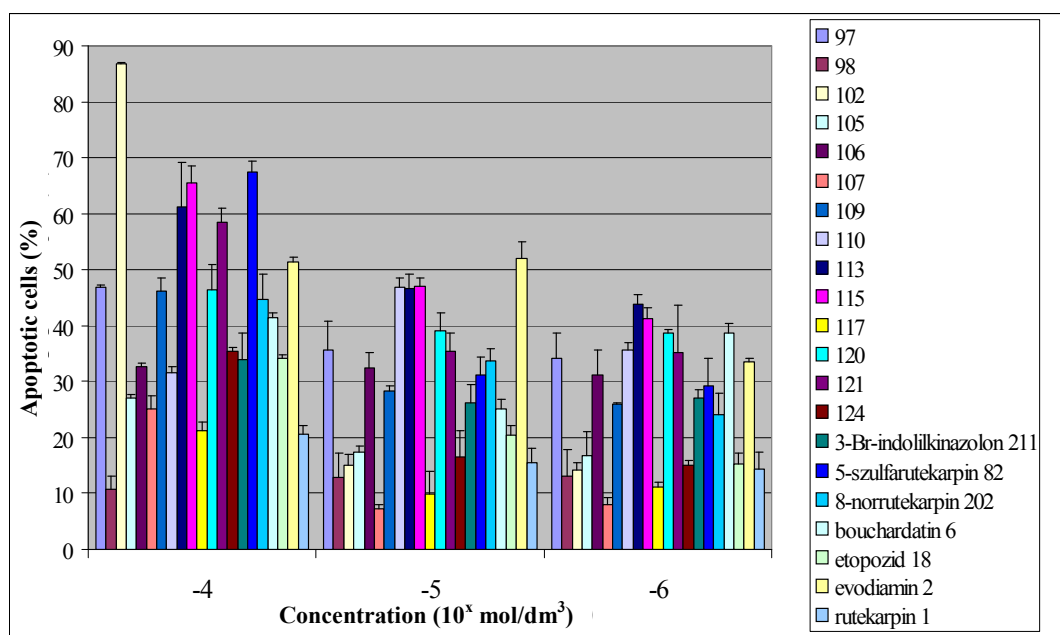


Figure 10. Proapoptotic effect of the tested compounds on HeLa cell line.

tioxidant effect). In our caspase-3 activation experiment none of our compounds showed significant effect, thus it is proven, that these exert their cytotoxic effect not via caspase activation. Structural comparison of the synthesized compounds and evodiamine implies that the reduced N-methyl structural element found in evodiamine might be necessary to trigger this mechanism. During the examinations with the help of the basic structure and substituents of effective compounds, we were able to draw conclusions regarding the directions of further development.

The caspase-activating effect in the case of the produced substituted hydrazone-derivatives is predictable by the results of Zhang et al, therefore these substances might be suitable as well for further pharmacological screening, and for production of A-ring substituted 8-norrutaecarpine-, 13-N-methyl-8-norrutaecarpine-, 14-N-methylrutaecarpine-, and 3-azarutaecarpine-derivatives.

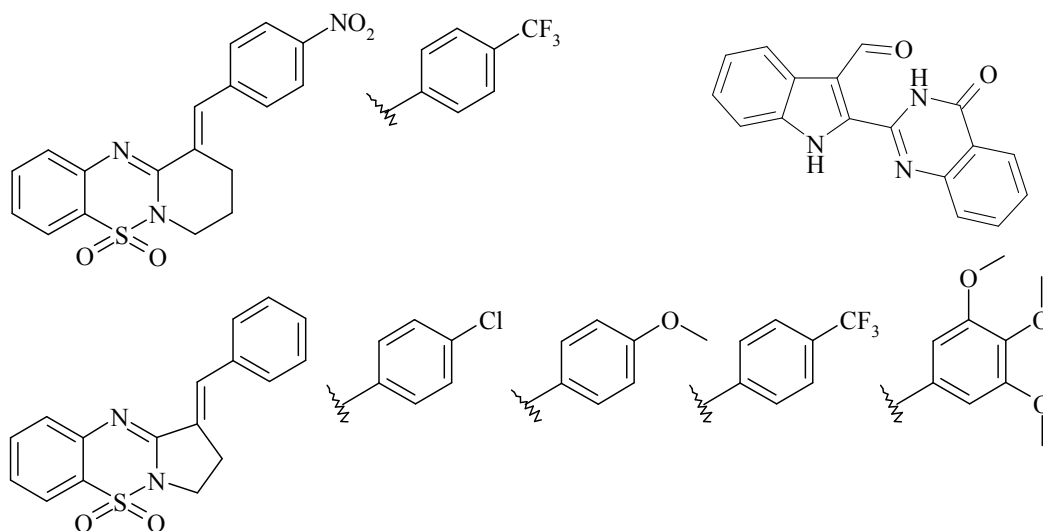


Figure 11. Compounds inducing nucleosomal fragmentation of HeLa cells at least by 30%

## References

### Publications of the thesis work

#### Original papers

1. **Bubenyák M.**; Pálfi M.; Takács M.; Béni Sz.; Szökő É.; Noszál B.; Kökösi J.  
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Independent citations: 4
2. **Bubenyák, M.**; Noszál, B.; Kóczian, K.; Takács, M.; Béni, Sz., Hermecz, I.; Kökösi, J.  
**Bioisosteric hybrids of two anti-inflammatory agents, rutaecarpine and piroxicam**  
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3. **Bubenyák, M.**; Takács, M.; Blazics, B.; Rácz, Á.; Noszál, B.; Püske, L.; Kökösi, J.; Hermecz, I.  
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**Synthesis of pentacyclic alkaloid hybrid**  
*Joint Meeting on Medicinal Chemistry (JMMC), Vienna, Austria* (lecture)  
20-23 June 2005.
2. **Bubenyák M.**; Kökösi J.; Noszál B.  
**Synthesis of quinazolinocarboline alkaloid analogues**  
*Joint Meeting on Medicinal Chemistry (JMMC), Portoroz, Slovenia* (poster)  
17-21 June 2007.
3. Szakács Z.; Béni Sz.; **Bubenyák M.**; Kökösi J.; Rácz Á.; Noszál B.  
**Protonation and Dynamics in Building Blocks of Benzothiadiazine Analogues of Anticancer Alkaloids**  
*SMASH Chamonix, Switzerland* (poster)  
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**Synthesis of quinazolinocarboline alkaloid analogues**  
*Congressus Pharmaceuticus Hungaricus XIV., Budapest* (poster)  
15 November 2009.

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1. Takács, M.; **Bubenyák, M.**; Váradi, A.; Blazics, B.; Horváth, P.; Kökösi, J.  
**Synthesis of novel ceramide-like penetration enhancers**  
*Tetrahedron Lett.* **2011**, *52*, 1863-1865.  
IF: 2,660

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