Boosting of NMR measurement methods and their usage in the pharmaceutical research for structure elucidation of small molecules in solution phase

Ph.D. Thesis

Sándor Boros

Semmelweis University
Doctoral School of Pharmaceutical Sciences

Consultants:	Katalin E. Kövér, D.Sc., member of HSA
	Gyula Batta, D.Sc.
	György Kéri †, D.Sc.
Official opponents:	János Rohonczy, Ph.D.
	Márta Kraszni, Ph.D.
Chairlady of the complex exam committee	Romána Zelkó, D.Sc.
Members of the complex exam committee:	Éva Szökő, D.Sc.
	István Antal, Ph.D.
	Mária Nikolics, Ph.D.

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1. Introduction

The NMR spectroscopic methods have been spectacularly developed since the beginning till now. At the beginning, the NMR characterization of the organic compounds consisted of only recording and description of ^{1}H- and ^{13}C-NMR spectra, as well as description of patterns of the signals, speculative analysis of the *J*(H,H) scalar coupling constants calculated from patterned signals.

Since the technological boom in the early 1990-s, the application of NMR spectroscopy has been more and more shifted to correlation techniques. The chemical bond network of the investigated molecules can be deduced from exact homo- and heteronuclear coupling networks set up by evaluation of two dimensional (2D) and selective one dimensional correlation spectra. The cross-relaxation phenomenon gives pieces of information about the 3D structure of the molecules, as well as the observable chemical exchanges inform us about the time-dependent behavior of the molecules.

The available high-field, high resolution and high sensitivity instruments as well as the multidimensional techniques significantly increased the size of the NMR-investigable molecules up to the biopolimers. However, we cannot forget about boosting of the NMR methods applied for determining the usual molecular size of *per os* administrated medicines.

2. Aims

During the NMR measurement, the phenomenon to be investigated cannot always be observed purely, but several times disturbing unwanted effects overlap with it. In other cases the phenomenon to be investigated is purposely planted onto another easily measurable one. The aim of the method development is to develop variants, which show the investigated phenomenon purely, truly, without disturbing effects, in order to get quantitative discursiveness. That is why filter and/or suppressor elements are built into the measurement sequences. Nevertheless, these cleaner elements should not cause new disturbing effects (instead of the eliminated ones), the decrease in the signal to noise ratio, the extreme elongation of the experiment time, and should not be sensitive for the small alterations to the experimental parameters and for the small deviations from the optimal parameters.

I processed in my theses five of my original publications. Two of them are first-author papers with topic of NMR method development; the development of the HSQMBC method available for measurement of heteronuclear long-range coupling constants as well as the zqs-easy-random-ROESY variant available for measurement of inter-atomic distances based on cross-relaxation phenomenon and chemical exchange processes. In both method developments, the comparison of spectra recorded on model compounds by the suggested and the formerly available variants proves the applicability of the suggested method. The application of the suggested method variants in practice are shown by investigation of samples of pharmaceutical research origin. These results were published in publications with topic of structure-activity-relationship. I confine myself to the presentation of only my own NMR spectroscopic measurements, but I intend to demonstrate, that the application of the suggested NMR spectroscopic methods were instrumental in the clarification of the chemical reaction pathways. I will include my observations on the time-dependent behaviors, micro-equilibriums of the investigated molecules.

The independency of the two method developments from each other determines the structure of the study, as the literature, experimental, evaluation and the practical application chapters are separated according to the two methods introduced. The results are resumed in a common summary.

3. Instruments and model compounds

The spectra of strychnine were recorded in the Chinoin Ltd. on a Bruker Avance II 400 NMR spectrometer, operating at 9.4 Tesla magnetic fields, equipped with a 5mm BBFO *z*-grad probe. The control of the instrument and the data processing was performed by TopSpin 2.1 software package. 5 mg strychnine base was solved in 0.6 ml deuteriochloroform. The spectra were recorded at 27 °C. The spectra of codeine and compound TT-232 were recorded in the Vichem Ltd. on a Bruker Avance 300 spectrometer operating at 7.04 Tesla magnetic fields, equipped with a 5 mm SEI *z*-grad probe. The control of the instrument and the data processing was performed by TopSpin 1.3 software package. 7 mg Codeine base as well as 10 mg TT-232-triacetate was solved in 0.6 ml deutero-dimethylsulfoxide, the spectra were recorded at 30 °C.

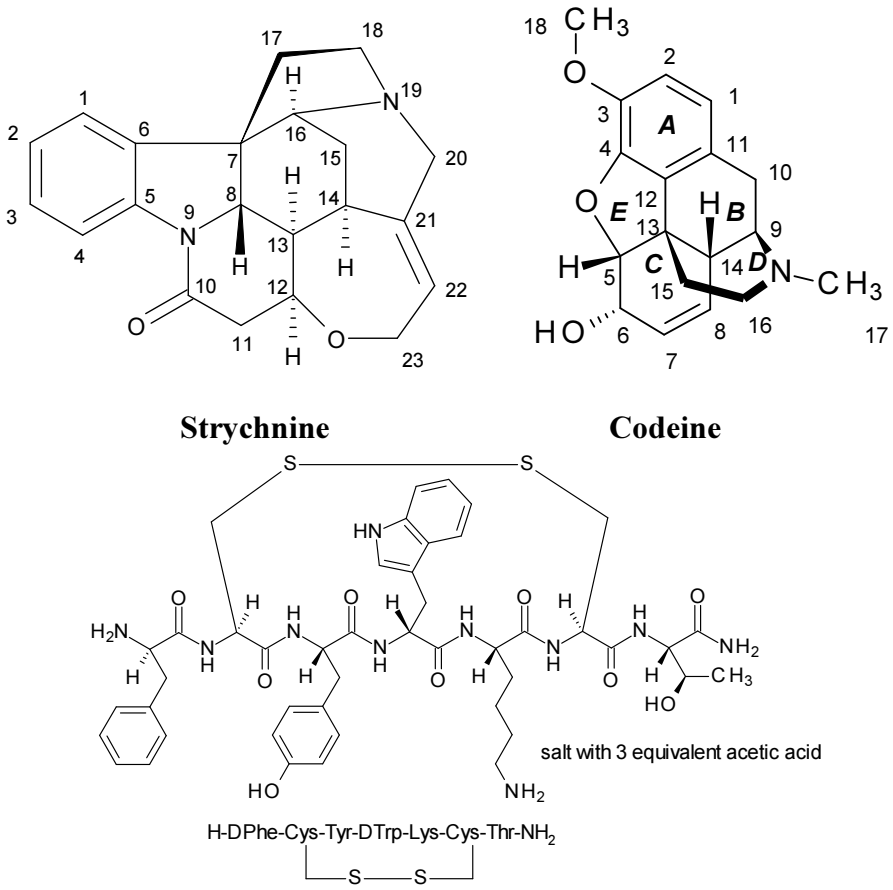


Figure 1. The chemical structure of the model compounds applied

Strychnine was used as a model compound for testing both boosted HSQMBC and ROESY variants. Codeine and TT-232 were used for the testing the boosted ROESY variant.

4. Results, theses

1.

I focused my research on the development of HSQMBC method available for precise measurement of heteronuclear long-range coupling constants. The result of my work is the low-power composite CPMG G-BIRD adiabatic HSQMBC variant.

This suggested version of HSQMBC incorporates several advantageous modifications of the original HSQMBC method developed in independent NMR schools. I adopted adiabatic inversion and refocusing pulses according from one source, G-BIRG CPMG filter block from another source, and from the third source the use of a high-power composite CPMG element

which is less sensitive for the minor inaccuracies of pulse calibration than the former variants, but it appreciably heats the sample and the electronics of the probe. My suggestion in the version developed with Katalin E Kövér (Debrecen University) is using the low-power of the composite CPMG element (grey backgrounded block in Fig. 2.) in order to reach higher safety of the electronics in the probe, as well as moving the position of the gradient blanking switch from the point between the last gradient pulse and the beginning of the acquisition to the point after the end of the acquisition in order to obtain a nicer signal shape. The suggested combination of the filter elements does not decrease the sensitivity of the method, does not increase experiment duration, but it is robust, i.e. it results in a version less sensitive for the small miscalibrations.

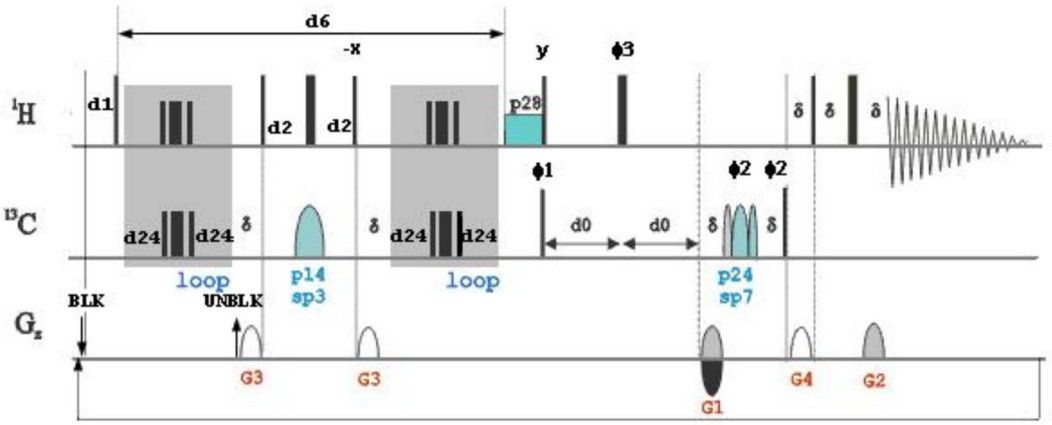


Figure 2. The scheme of the suggested low-power composite CPMG G-BIRD adiabatic HSQMBC version. The thin and thick bars represent 90 as well as 180° high-power pulses. The shorter thin and thick bars before grey background represent the low-power filter pulses. The blue half-ellipses represent the adiabatic inversion ad refocusing pulses. d24 = 120-180 μsec. Gradient pulses: G1 : G2 : G3 : G4 = 80 : 20.1 : 17 : 11 %.

The efficiency of the suggested method is demonstrated on comparison measurements using strychnine as a model compound. [**I**]

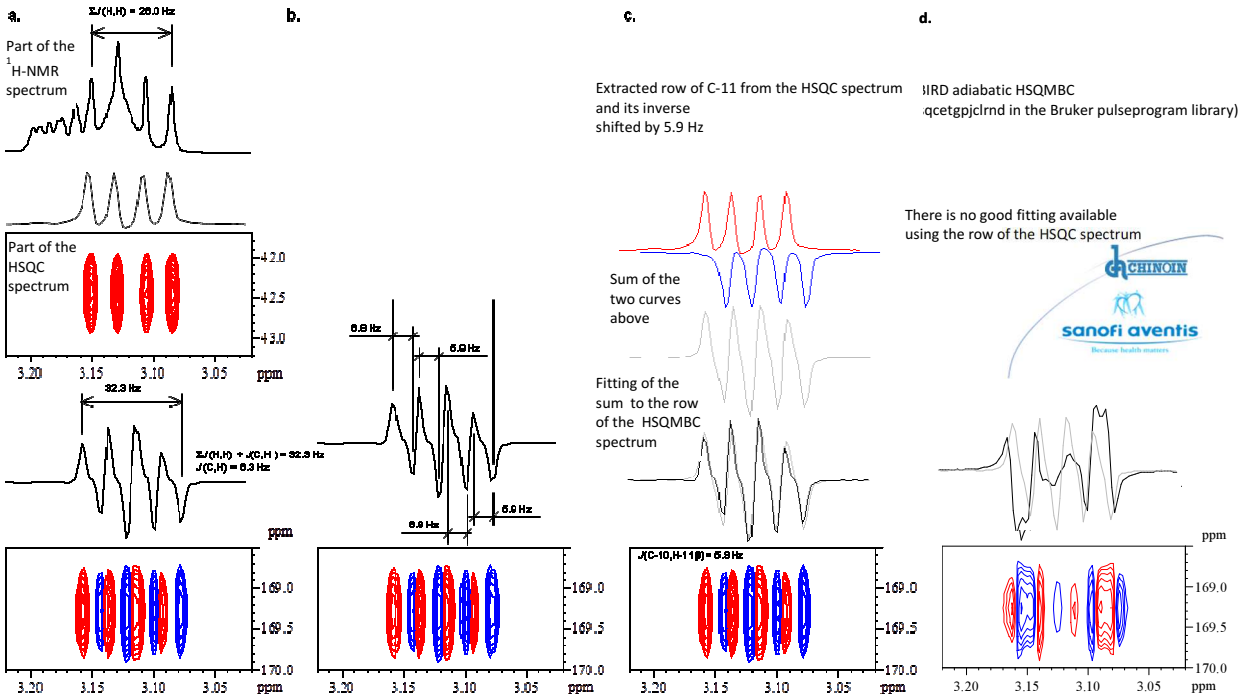


Figure 3. Methods for extraction of the heteronuclear long-range coupling constant from the HSQMBC a.) based on the distance of the winger spectral lines. b.) based on the distance of the corresponding opposite-phase spectral lines. c.) using spectrum simulation. d.) unsuccessful attempt of fitting of simulated signal shape to the spectrum element recorded by the method version available in the Bruker pulseprogram library

2.

I also prepared and tested the selective 1D version of the low-power composite CPMG adiabatic HSQMBC. [**I**]

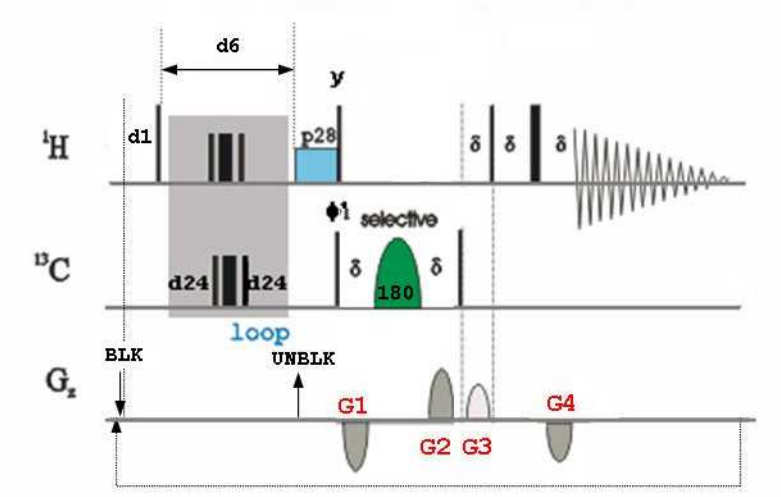
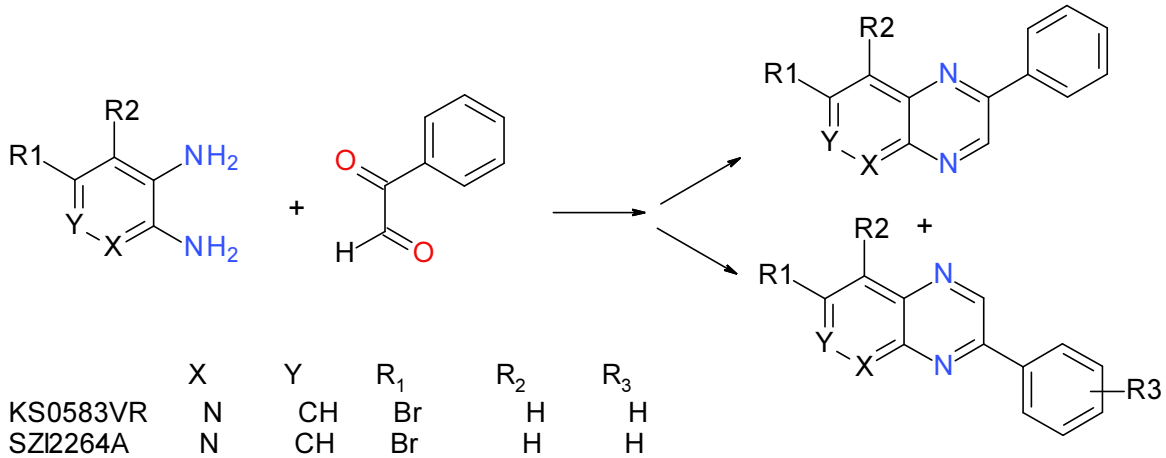


Figure 4. Scheme of the suggested selective 1D version of the low-power composite CPMG adiabatic HSQMBC. The green half-ellipse represents the selective 180°pulse. d24 = 120-180 μsec, Suggested values for the gradient pulses: G1 : G2 : G3 : G4 = -40 : 40 : 10 : -20 %

3.

The suggested HSQMBC variant was successfully applied in the Vichem Ltd. for discrimination of formed, differently substituted quinoxaline and pyridi-pyrazine isomers. [**II**, **III**]



	X	Y	R_1	R_2	R_3	
KS0583VR	N	CH	Br	H	H	
SZI2264A	N	CH	Br	H	H	
JJ1677X15	CH	CH	NO_2	H	meta-(3-trifluorometil-benzoil-amido)	
LA123X6	CH	N	H	H	H	
LA182X1	CH	CH	H	F	H	

Figure 5. Synthesis of quinoxaline and pyrido-pyridine derivatives with two possible products.

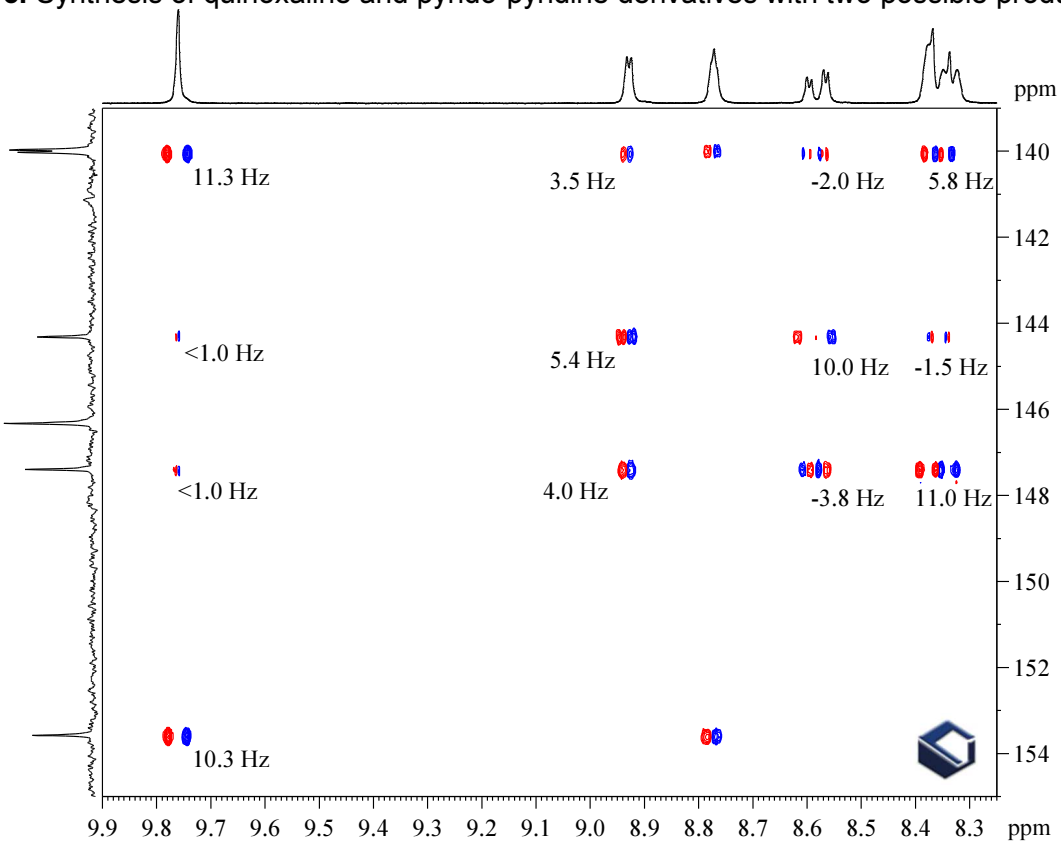


Figure 6. Section of the HSQMBC spectrum of compound **JJ1677X15**. Digital resolution: 0.3 Hz/pt.

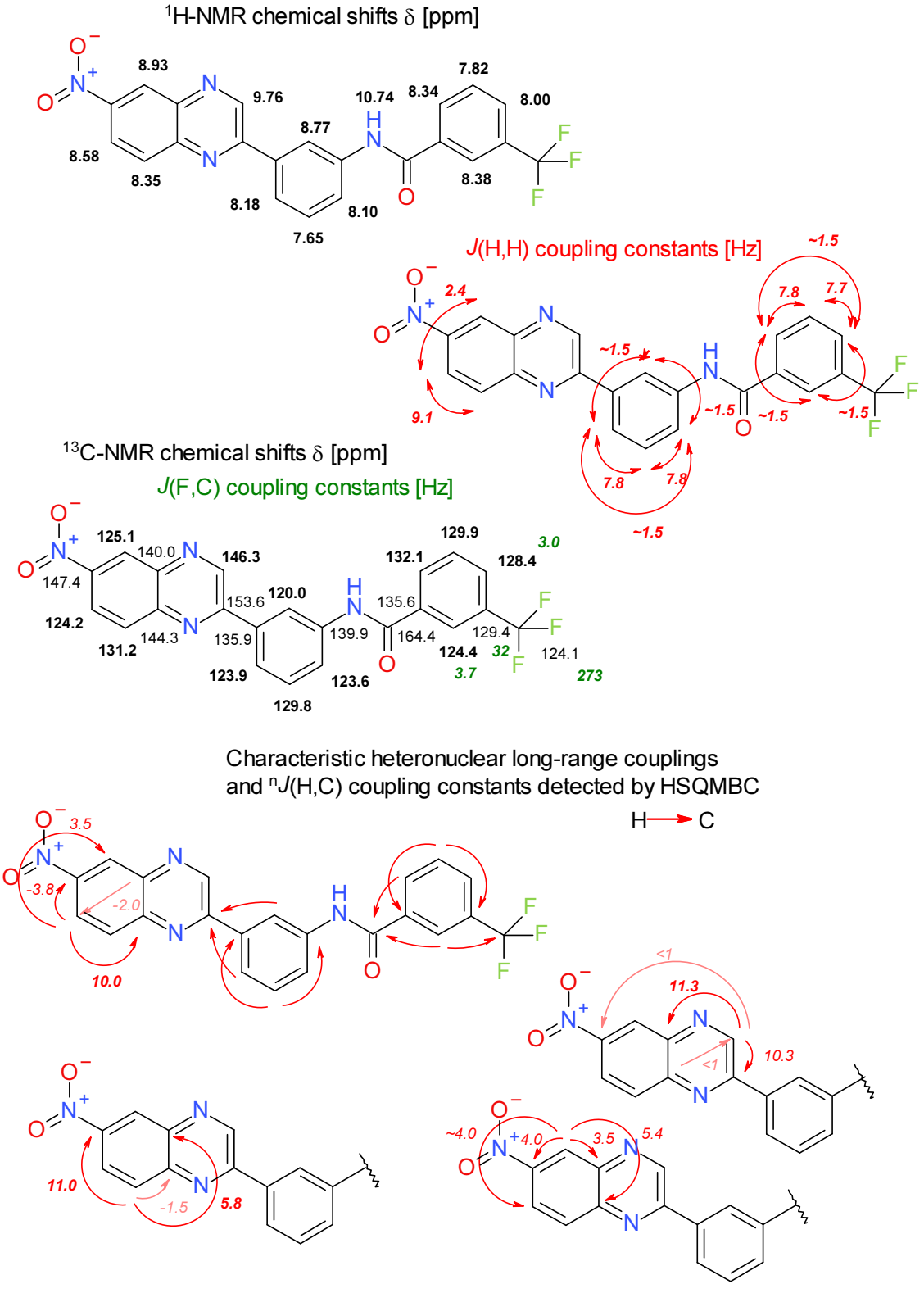


Figure 7. Evaluation of the spectra of sample **JJ1677X15**; ^{1}H- and ^{13}C-NMR signal assignment, as well as the interpretation of the heteronuclear long-range coupling constants. The values of heteronuclear long-range coupling constants are depicted only on the crucial part of the structure.

4.

Initially in Chinoin Ltd., later in Vichem Ltd., with prof. Gyula Batta (University of Debrecen) I developed the zqs-easy-ROESY method for detection of intermolecular ^{1}H-^{1}H steric proximities and chemical exchange processes. The adiabatic inversion and simultaneous mild gradient pulses filter element suggested for TOCSY and NOESY measurements were built into the off-resonance version of ROESY (easy-ROESY). The suggested zqs-easy-ROESY variant is efficiently usable in wider region incorporating the smaller molecular mass region than the former ROESY versions.

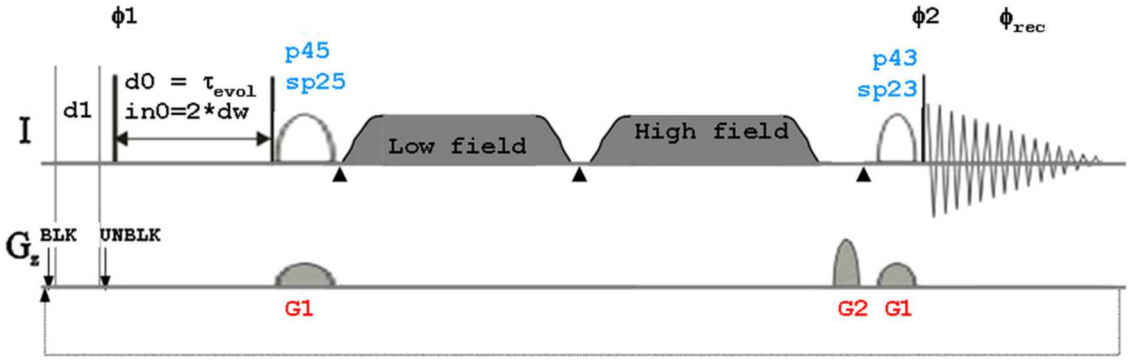


Figure 8. The scheme of offset compensated zero-quantum suppressed adiabatic ROESY (zqs-easy-ROESY) sequence.
The thin bars represent hard 90° pulses. The two unfilled half-ellipses represent the adiabatic shaped inversion pulses. The suggested duration of the inversion pulses is 50 and 30 ms, respectively. The duration of the half Gaussian ramp pulses before and after the spin-lock pulses is 1 ms. The duration of the spin-lock periods is twice 100-150 ms depending on the molecular mass of the investigated compound. The spin-lock time is randomizable by ±5%. ▲ marks represent the positions of the frequency switching between the on-resonance, up-tuned and down-tuned off-resonance stages.

The efficacy of the suggested method was tested by comparative measurements of codeine, strychnine and TT-232 compounds. The method was validated by statistic evaluation of the measurements on codeine and strychnine [**IV**, **VIII**]. Figure 9 shows the overview spectrum of strychnine recorded by the suggested zqs-easy-ROESY variant.

Figure 10 shows selected cross-peaks from spectra recorded by different method versions. The left part of the figure shows selected cross-peaks which are also coupling partners. The phase alternation of the signals due to zero-quantum effects is visible on the signals recorded by the former versions, as well as pure positive phase is shown on the signals recorded by the suggested version. The right part of the figure shows cross-peaks which are not signals of coupling partners, therefore there are no signal in the COSY spectrum, but these signals are close to the main diagonal of the spectrum. The cross-peaks of the first line are satisfactory recorded by

either version. The second and third as well as the fourth and fifth lines show the signals of the same steric proximity on the opposite sides of the main diagonal. The cross-peak in the suggested version is integrable even nearby the main diagonal, as well as nearby an alternating-phase signal, too, in contrast with the former methods where these signals are ambiguous.

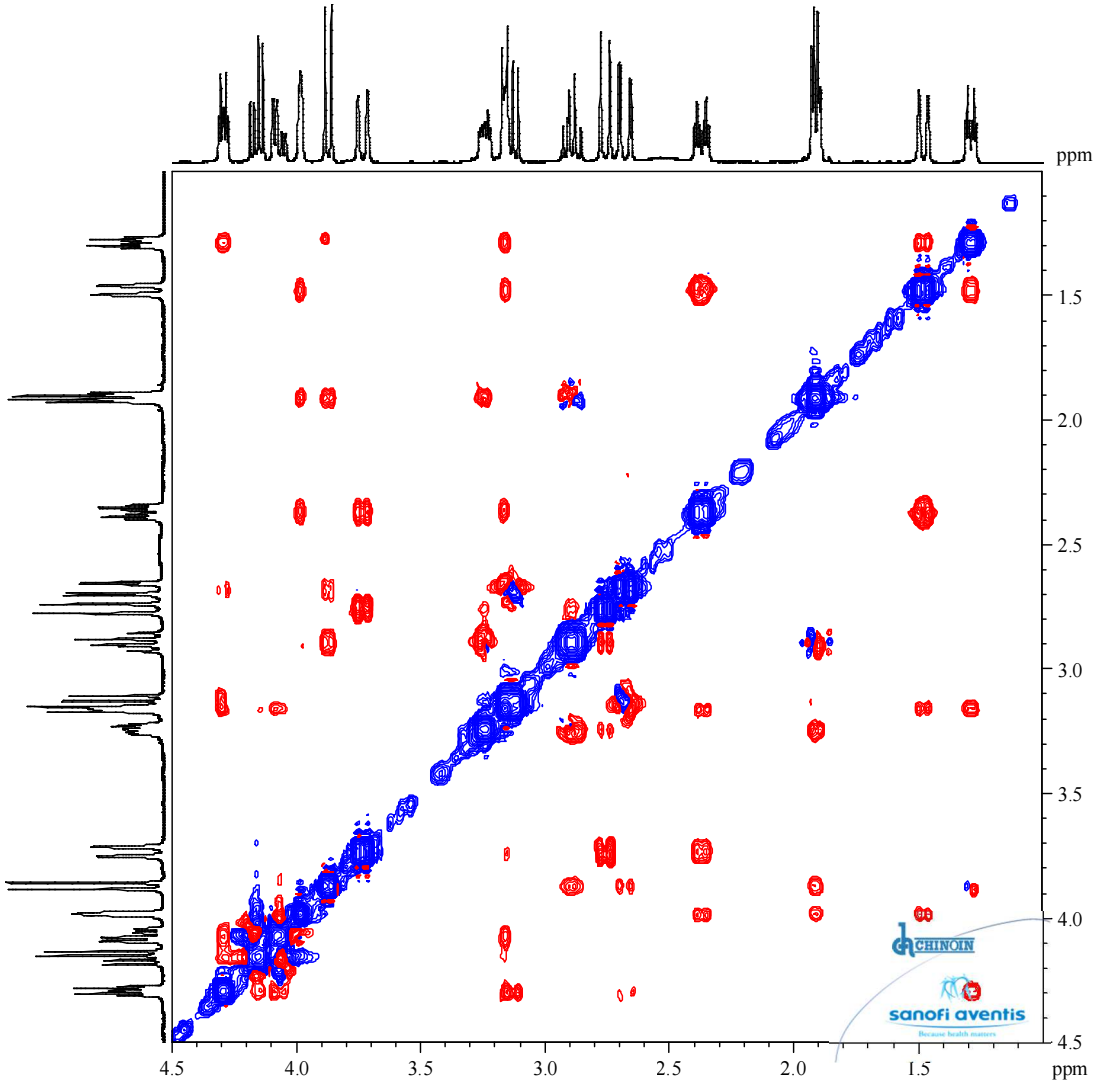


Figure 9. Offset-compensated zero-quantum suppressed (zqs-easy-) ROESY spectrum of compound strychnine. [**IV**]

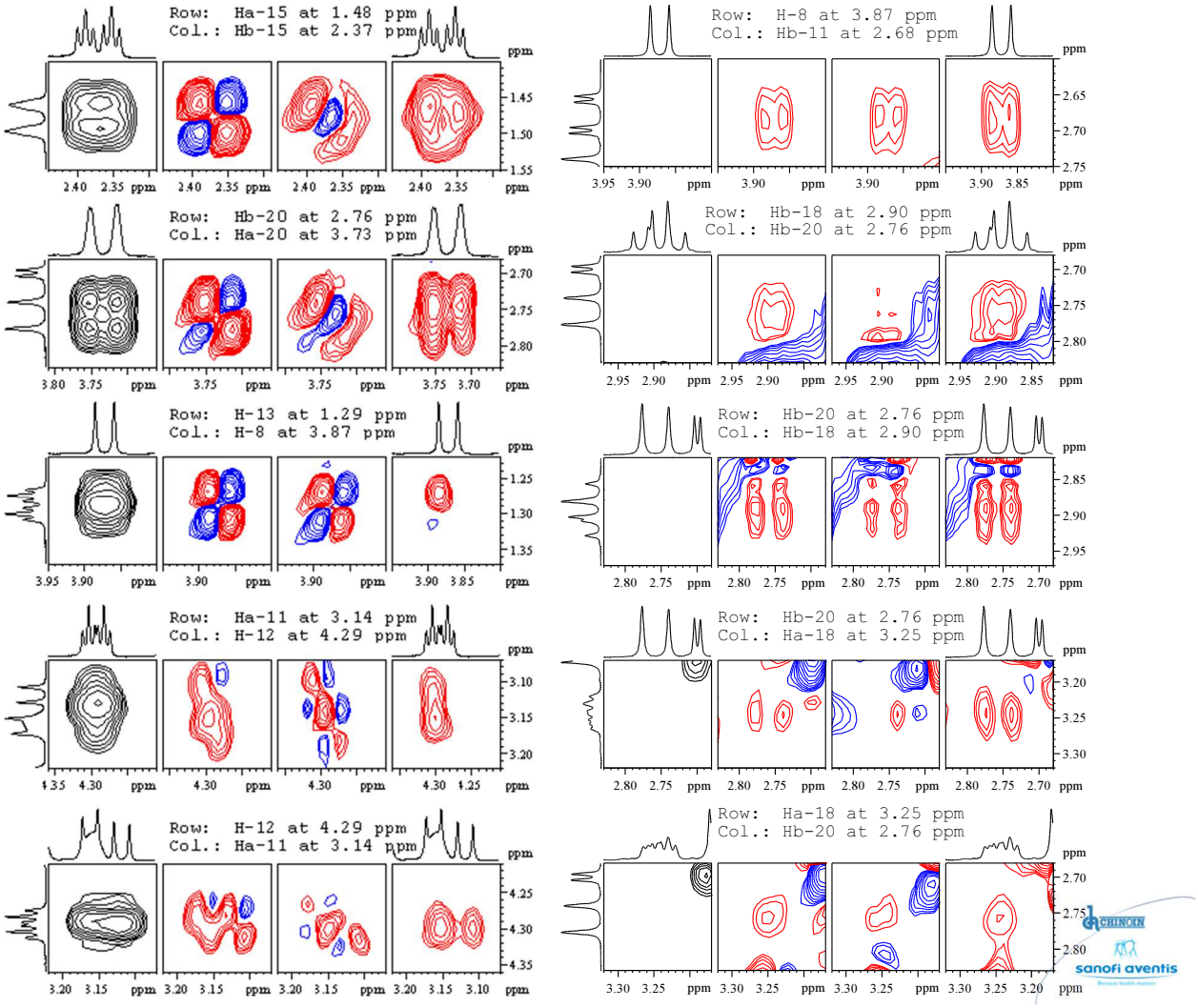


Figure 10. Selected cross-peaks from the ROESY spectra of compound strychnine.
The first column contains signals from the COSY spectrum, the second one is recorded by the ROESY version available in the Bruker pulse-program library, the third one is recorded by the easy-ROESY available in the literature, and the fourth one is recorded by my own version.

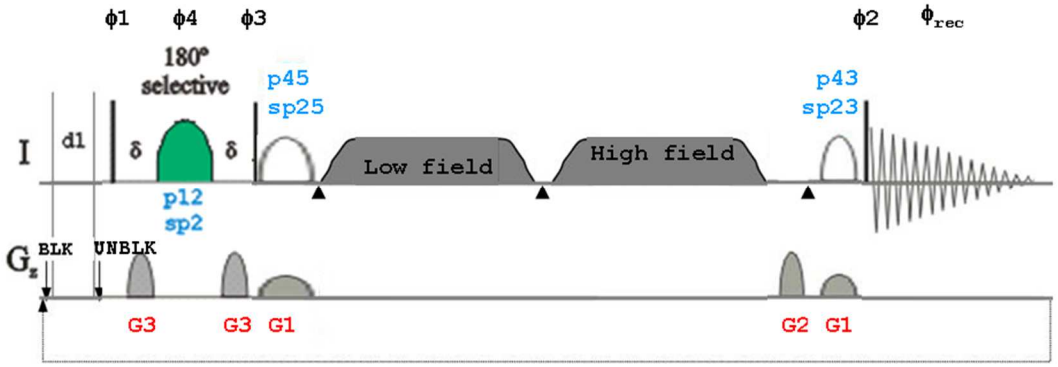


Figure 11. Scheme of the suggested single pulse field gradient selective excitation selective 1D zero-quantum suppressed efficient adiabatic symmetrized (spfge-se-1D-zqs-easy) ROESY pulse sequence.

5.

The zqs-easy-ROESY version was successfully applied for investigation of intermediates and final products containing imidazo-pyiridine and imidazo-pyrazine core structure. [**V**] The synthesis required strict NMR spectroscopic control because some intermediates contained two reaction centers in the molecule for the following reaction step, e.g. in step c., where a halogenic atom was changed to an amine group, whereas the molecule contained another halogenic atom, too, as well as in step e. and g., formation an amide or urea moiety, where the intermediate contained two acylable amide groups.

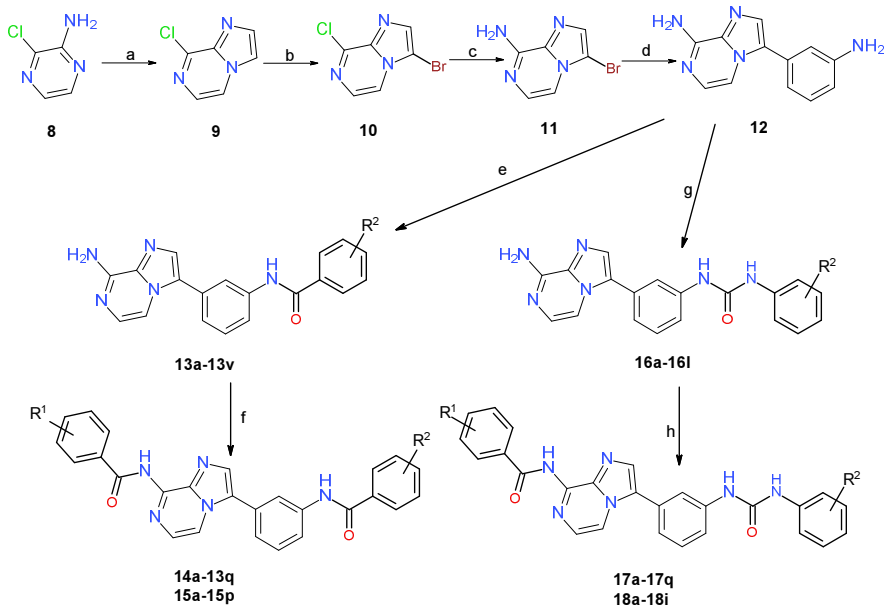


Figure 12. Manufacturing of compounds containing imidazo[1,2-*a*]pyrazine core structure.

6.

Tautomeric equilibrium was observed during the investigation of final compounds containing imidazo-pyrazine core structure. The ^{1}H-NMR signals of a well-defined region of the final compounds containing imidazo[1,2-*a*]pyrazine core structure are broad, while the spectra of the analogous pyrido-pyrazine derivatives are regular. The reason of this behavior is that there are unusual tautomeric forms of the benzoic-amide moiety bonded to this ring system which are now stabilized by intramolecular H-bond. This stability requires the nitrogen atom at position of 7 in the imidazo[1,2-*a*]pyrazine core structure.

The synthetic observations show that while the imidazo[1,2-a]pyridin-8-benzamide structure can be built by Suzuki reaction toward to the final product, the amide bond of the imidazo[1,2-a]pyrazin-8-benzamide structure decomposes during the conditions of Suzuki reaction. That is

why the building of theoretically analogous structures had to be performed using different synthetic routs. The line broadening of some characteristic signals in the ^{1}H-NMR spectrum of imidazo[1,2-a]pyrazin-8-benzamide as well as the unexpected decomposition of its amide bond are in correspondence with the presence of unusual tautomers containing H-bond attenuating the amide bond in the molecule. [**V**]

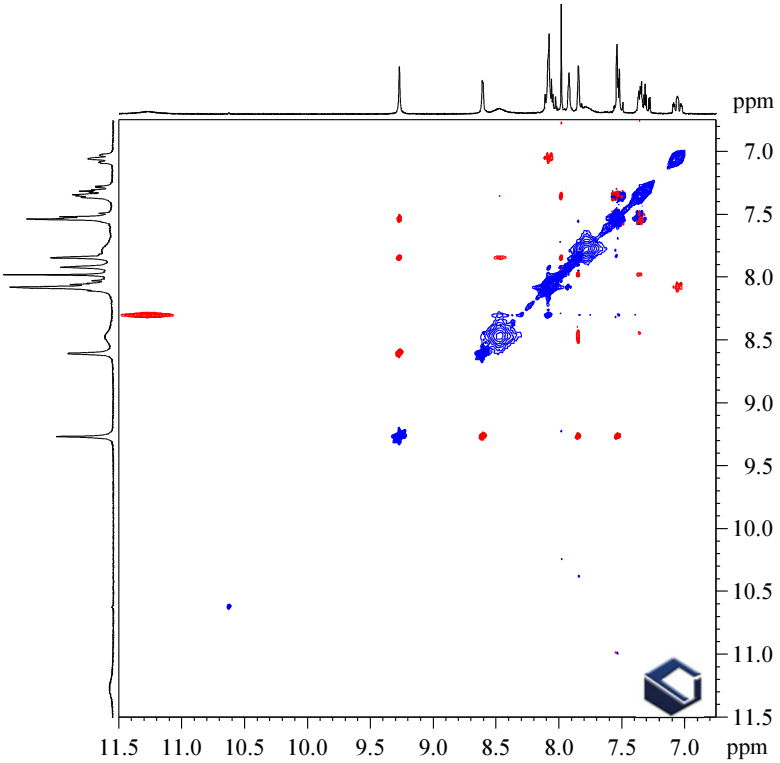


Figure 13. zqs-easy-random-ROESY spectrum of compound **VIC31874 (RG1505)**.

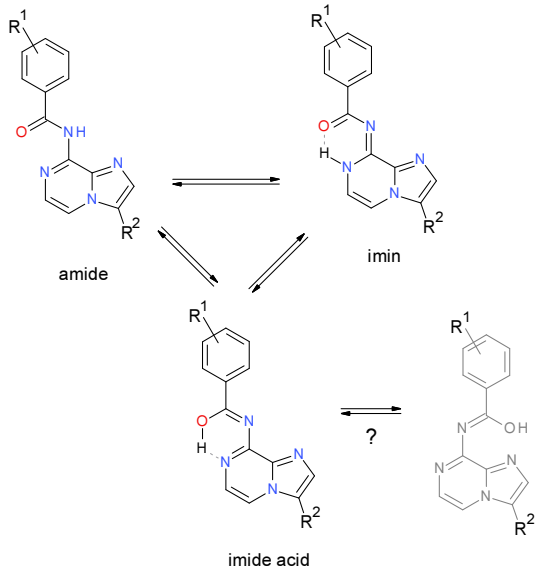


Figure 14. Tautomeric equilibral forms of the imidazo[1,2-a]pyrazin-8-benzamide ring systems. When the benzoyl group contains electronegative *ortho* substituent, the amide form is stabilized.

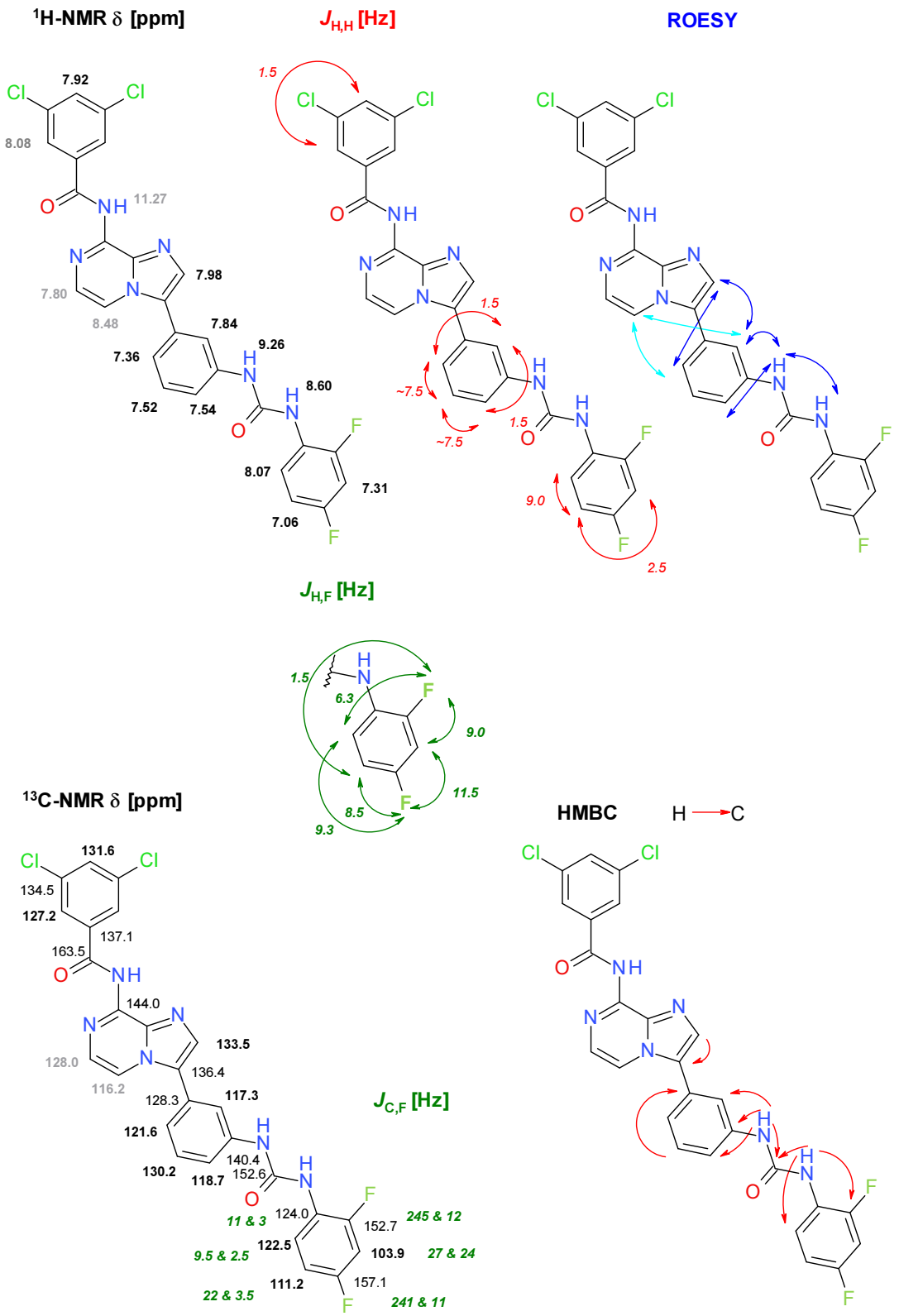


Figure 15. Interpretation of the NMR spectra of compound **VIC31874**, sample **RG1505** (compound **17e** in **V**). The chemical shifts depicted by grey color represent the region of the molecule where the signals are broad in the ^{1}H- and ^{13}C-NMR due to the tautomeric equilibrium.

7.

An unexpected methyl group migration was observed and proven by NMR spectroscopic methods, during formation of pyrido[2,3-d]pyrimidin core structure. Carboxylic acid methyl ester was applied as reactant, the methyl group was identified as bonded to the nitrogen atom in the product. [**VI**, **VII**]

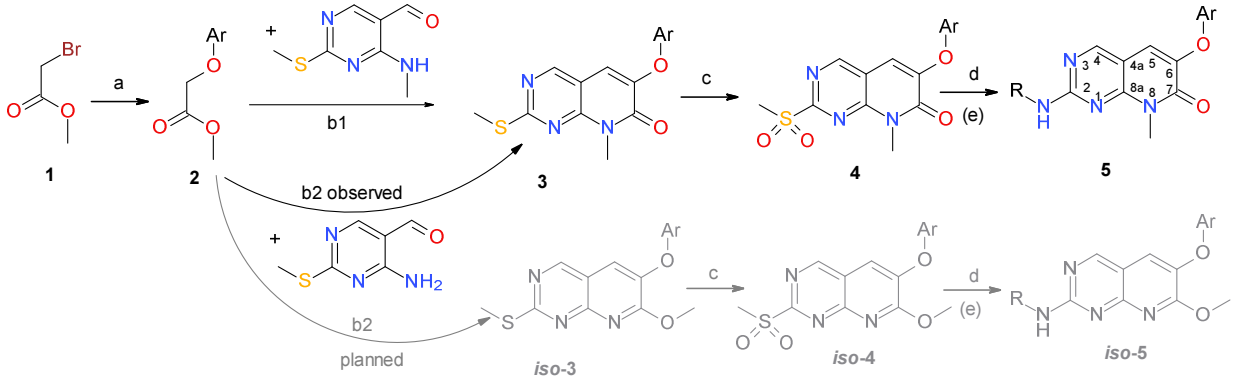


Figure 16. Planned synthesis of 2-alkylamino-6-aryloxy-7-methoxy-pyrido[2,3-d]pyrimidin derivatives and observed formation of 2-alkylamino-6-aryloxy-8-methyl-pyrido[2,3-d]pyrimidin-7-one derivatives via two routes. Gray color represents the planned but not realized synthetic rout.

8.

The ^{1}H- and ^{13}C-NMR spectra of the prepared final products having pyrido[2,3-d]pyrimidin-7-one core structure contain two signal sets with ratio 70:30. The corresponding signal pairs give EXSY cross-peaks in the NOESY spectrum as well as these pairs coalescence at higher temperature. The rationale of the phenomenon is that the realistic structure and charge distribution should be taken account of, where there is a partial double bond between the ring and the *exo* nitrogen atom, the electronegative atoms of the core have partial negative as well as the H atom of the secondary amine group represents a partial positive charge. The restricted rotation requires the presence of the carbonyl group in the core structure. The rotation is free in 2-alkylamino-6-aryloxy-pyrido[2,3-d]pyrimidin as well as 2-dialkylamino-6-aryloxy-8-methyl-pyrido[2,3-d]pyrimidin-7-one derivatives. [**VI**, **VII**]

The restricted rotation of the tertiary amides is well known, but the restricted rotation of the secondary amines is a very infrequent phenomenon observable only in very special chemical conditions and structures.

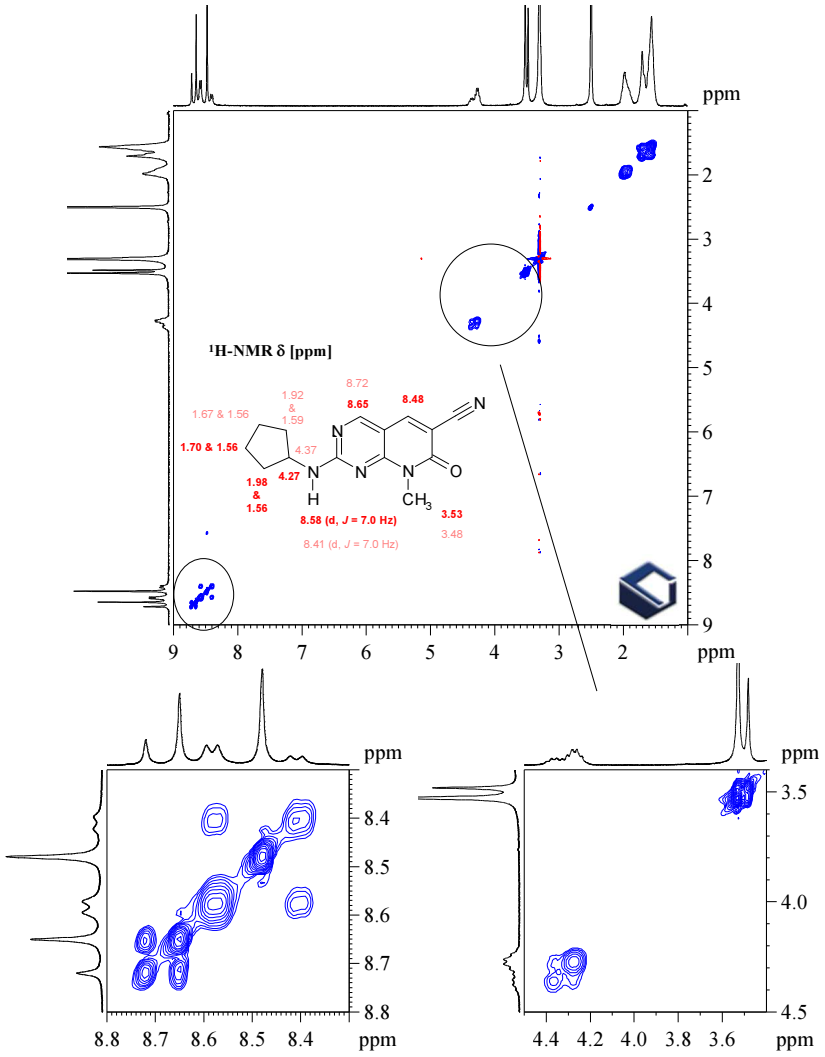


Figure 17. NOESY (EXSY) spectrum of 6-cyano-2-cyclopentylamino-8-methyl-pyrido[2,3-d]pyrimidin-7-one model compound (τ_{mix} = 250 ms) as well as expanded regions of the spectrum

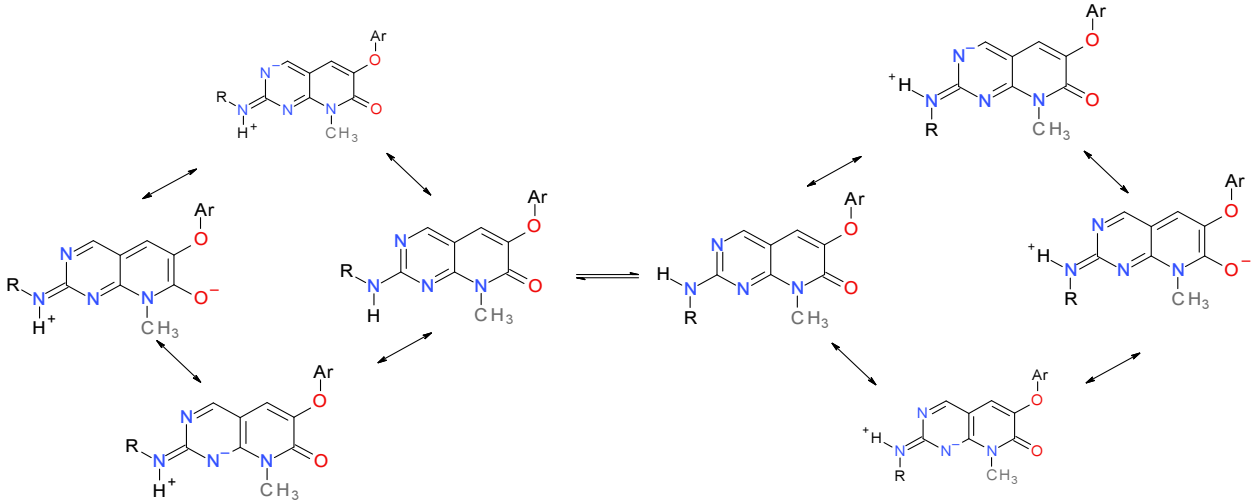


Figure 18. Rotameric equilibrium and contributing mesomeric structures of 2-alkylamino-6-aryloxy-8-methyl-pyrido[2,3-d]pyrimidin-7-one derivatives.

List of own publications:

I.

Sándor Boros, Katalin E. Kövér:

Low-power-composite-CPMG HSQMBC experiment for accurate measurement of long-range heteronuclear coupling constants

Magn. Reson. Chem., **49**, 106-110 (2011) DOI: 10.1002/mrc.2717

II.

László Kékesi, András Dancsó, Eszter Illyés, **Sándor Boros**, János Pató, Zoltán Greff, Gábor Németh, Rita Garamvölgyi, Ferenc Baska, László Örfi, György Kéri:

Preparation of Pyrido[2,3-b]pyrazine Ring System via Regioselective Condensation Reaction

Lett. in Org. Chem., **11**(9), 651-656 (2014)

DOI: 10.2174/15701786116661406062050 28

III.

Kékesi László, Sipos Anna, Németh Gábor, Dancsó András, Illyés Eszter, **Boros Sándor**, Breza Nóra, Nemes Zoltán, Hegymegi-Barakonyi Bálint, Pató János, Greff Zoltán, Kéri György, Őrfi László:

Erlotinib-érzékeny és erlotinib-rezisztens sejtvonalakat gátló pirido[2,3-b]pirazinok, és előállításuk régiószelektív kondenzációs reakcióval

Acta Pharmaceutica Hungarica, **84**, 91-104 (2014)

IV.

Sándor Boros, Gyula Batta:

Offset-compensated and zero-quantum suppressed ROESY provides accurate $^{1}H–^{1}H$ distances in small to medium-sized molecules

Magn. Reson. Chem., **54**, (12), 947-952 (2016) DOI: 10.1002/mrc.4474

V.

Rita Garamvölgyi, Judit Dobos, Anna Sipos, **Sándor Boros**, Eszter Illyés, Ferenc Baska, László Kékesi, István Szabadkai, Csaba Szántai-Kis, György Kéri, László Őrfi:

Design and synthesis of new imidazo[1,2-*a*]pyridine and imidazo[1,2-*a*]pyrazine derivatives with antiproliferative activity against melanoma cells

Eur. J. Med. Chem., **108**, 623-643 (2016) DOI: 10.1016/j.ejmech.2015.12.001

VI.

Laura Simon-Szabó, Márton Kokas, Zoltán Greff, **Sándor Boros**, Péter Bánhegyi, Lilián Zsákai, Csaba Szántai-Kis, Tibor Vántus, József Mandl, Gábor Bánhegyi, István Vályi-Nagy, László Őrfi, Axel Ullrich, Miklós Csala, György Kéri:

Novel compounds reducing IRS-1 serine phosphorylation for treatment of diabetes

Bioorg. Med. Chem. Letters, **26**, 424-428 (2016) DOI: 10.1016/j.bmcl.2015.11.099

VII.

Kéri György, Őrfi László, Greff Zoltán, Bánhegyi Péter, Szántai-Kis Csaba, Erős Dániel, **Boros Sándor**, Breza Nóra, Zsákai Lilián:

Novel use of kinase antagonist compounds to alter fibrotic cell proliferation

(Új kináz inhibitor hatású vegyületek, és felhasználásuk fibrotikus sejtosztódás szabályozására)

P1500620 Hungarian patent application, (Dec. 16., 2015.)

VIII.

Sándor Boros, Zoltán Gáspári, Gyula Batta:

Accurate NMR Determinations of Proton–Proton Distances

In *Annual Reports on NMR Spectroscopy* (ed. by G. A. Webb), **94**, 1-39 (2018)

DOI: 10.1016/bs.arnmr.2017.12.002