STRUCTURE ELUCIDATION OF BIOACTIVE MOLECULES BY APPLYING MODERN NMR TECHNIQUES

PhD thesis

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DEBRECEN, 2001

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I. Preliminaries and the aim of the thesis

The subject of this thesis is the structural study of biologically active compounds, proline derivatives, flavonones and benzofuranones.

My dissertation was based on the work executed by me as a member of a team. The organic molecules presented in chapters "Investigations on the prolyl-endopeptidase (PEP) inhibitors by NMR techniques" and "Analysis of 3(2*H*)-benzofuranone derivatives with NMR methods" were prepared in Chinoin Pharmaceutical and Chemical Works Ltd. under supervision of Dr. Sándor Bátori, Dr. Ágnes Horváth and Dr. Károly Kánai. Organic molecules referred to in chapter "Configuration and structure elucidation of 3-substituted flavanone derivatives with NMR methods" were synthesized by myself at the Department of Organic Chemistry of Debrecen Kossuth Lajos University under supervision of Dr. György Litkei and Prof. Dr. Sándor Antus. During the structural study of these compounds my aim was to gain detailed data including the relative configuration and conformation of molecules investigated. This information broadened our understanding the mechanisms of the formation of the compounds.

Prolyl-endopeptidase (PEP) inhibitors:

As a member of serine protease family PEP is a large (about 80 kDa) intracellular serine protease, which consists of 710 amino acids and has monomer structure. PEP preferentially hydrolyses proline-containing peptides at the carboxy end of proline residues. This enzyme is not related to the well-known *chymotrypsin or subtilisin* family of enzymes, but represents a new family of serine proteases. Specifically, the order of the residues of the catalytic triad is different in the three protease families: *His-Asp-Ser* in *chymotrypsin*, *Asp-His-Ser* in *subtilisin*, and *Ser-Asp-His* in prolyl oligopeptidase. Furthermore, the PEP differs from other serine proteases by lack of the free NH group in its structure.

Prolyl-endopeptidase discovered in 1971 is responsible for *Pro-Lys* bond cleavage in *oxytocin*. It was found that PEP is widely distributed in mammals and can be purified from various organs including brain (*hyppocampus* and *cerebral cortex*). This enzyme is found both in *cytosolic* and *membrane* bound forms.

Many proline-containing neuropeptides and hormones may appear as endogen substrate of PEP. For example, *Substance P* (*H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2*), *vasopressin-arginin* (*NH_2-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2*) neuropeptides and *TRH* (*Thyreotrop-Releasing Hormon: pGlu-His-Pro-NH_2*) play an important role in changing of the performance in learning and memory tasks in both humans and animals. Therefore, inhibitors of PEP have been proposed as targets for therapeutic intervention to treat and prevent diseases with associated memory loss, such as Alzheimer's disease and senile dementia.

Due to above mentioned important role of PEP on the influence of memory tasks its inhibitors can be used as efficient pharmacon to cure different neurodegenerative diseases or sometimes to prevent from them. So, in 1993 we started our research for new PEP inhibitors at R&D Department of Chinoin Pharmaceutical and Chemical Works Co. Ltd. Determination of 3D structure of the PEP had been also included in the project and was successfully accomplished in 1998.

We set a dual goal when researching the prolyl-endopeptidase inhibitors: - on the one hand the determination of the conformation of the well-known PEP inhibitor molecule coded as SUAM-1221 in solution and in parallel on the other hand the research and development of new molecules more effective than the known inhibitors.

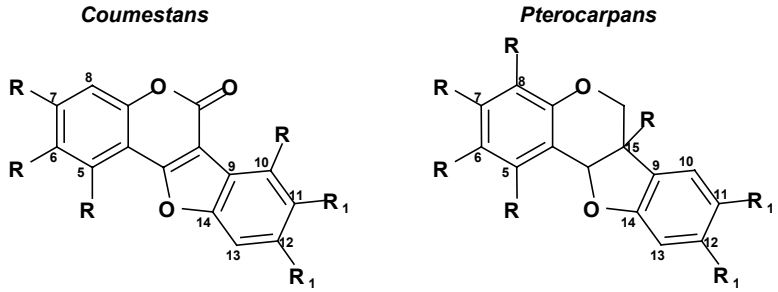
Flavonoids and benzofuranones:

Due to their biological influence flavonoids have been widely investigated in past decades by numerous researchers. Szent-Györgyi and his followers studied first the biological and pharmacological activity of these natural products. Today, several new synthetic flavonoid derivatives are possessing definite effect (Venoruton, flavanone-8-acetic acid).

Since decades, the synthesis of new flavonoid derivatives is one of the main projects at Department of Organic Chemistry of Debrecen University. The research in this project is important not only from the theoretical point of view, but it also has practical hints to obtain new pharmacons. Because of many different kinds of the pharmacological activity of 6-aryl-pyridazinones, intense work has been in progress since 1970 in conjunction with these compounds. Thus, beside their influence on blood pressure, similarly to heart glycosides they have remarkable positive inotrop effect. Several synthetic medicines have already been lunched on the market (Amrinon, Mibrinon, Imadozon). Recently pyridazinone derivatives have been obtained from quinolinones, tetralones and chromanones. Synthesis of new

pyridazinone derivatives and its intermediates from flavanones and their structure elucidation were the next goal of this thesis.

The investigation of natural bioactive flavonoids and structural related compounds become more and more significant because of their growing therapeutic importance. The newly synthesized derivatives often contain the same heteroaromatic groups as the natural flavonoids have. The furan and benzofuran as substructure occur in aurone derivatives, in carnation oil, in seeds of flowers of chrysanthemum and hydrangea, in different kinds of citruses and in several alkaloids. Moreover, the *coumestans* and *pterocarpans* belonging to the family of isoflavones also contain a benzofuran part in their structure.



Ipriflavone, one of the synthetic isoflavones, has been applied in the therapy of osteoporosis for decades. Ipriflavone is the active ingredient of *Osteochin*®, the original drug lunched to the market by Chinoin Chemical and Pharmaceutical Works Co. Ltd. Ipriflavone has an effect on the mithochondrial oxidative phosphorilation, so it increases the utilization of oxygen by cells. Ipriflavone is supposed to inhibit the bone reduction and also to influence on bone formation by increasing the calcium retention of bone cells. In the Chinoin Chemical and Pharmaceutical Works Co. Ltd. new isoflavones or related compounds have been investigated in the course of Osteochin research project. Since several isoflavonoids contain benzofuran or its analogues as subunit we aimed the synthesis of 3(2*H*)-benzofuranone derivatives and the determination of their structure by application of different NMR methods.

II. Applied methods

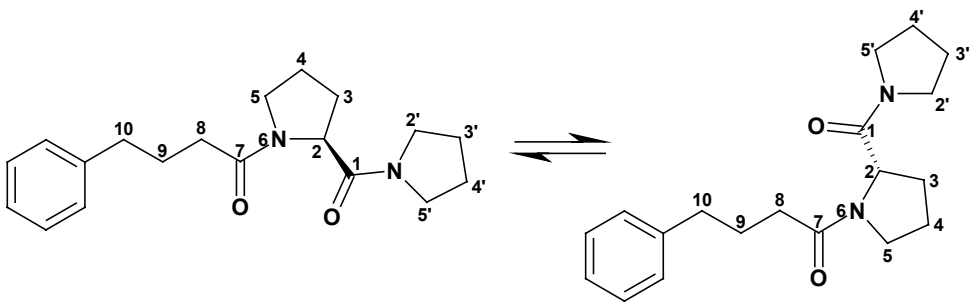
During the synthetic work the classical preparative organic chemical methods have been applied. The monitoring of the reactions and purity of the obtained compounds were checked by TLC. The purification of the crude products as well as separation of formed isomers was done by applying recrystallization and column chromatography.

The structure elucidation of newly obtained molecules and compounds formed according to the literature was proved primary on the bases of the NMR measurements (BRUKER WP-200SY, BRUKER DRX-400). Additionally, elemental analysis data, IR, MS and single crystal X-ray diffraction were also used. The NMR measurements were executed on the samples dissolved in different deuterated solvents. The assignment of the ^{1}H and ^{13}C NMR signals was based on the 1D and 2D standard and gradient NMR experiments, such as: DEPT-135, selective INEPT, ^{1}H-^{1}H 1D and 2D COSY, ^{1}H-^{1}H 1D difference NOE, ^{1}H-^{1}H 1D(gradient) and 2D NOESY, ^{1}H-^{1}H 1D(gradient) and 2D TOCSY, ^{13}C-^{1}H 2D HMQC, ^{13}C-^{1}H 2D HMBC, 2D*J*-resolved spectroscopy, ^{1}H and ^{13}C T_1 relaxation time determination. Besides the measurements mentioned above the ASIS (*A*romatic *S*olvent *I*nduced *S*hifts) NMR method was also used.

Conformational analysis of molecules investigated has also been performed using molecular mechanic calculations (MM^{+}, HyperChem software package). In case of SUAM-1221 both molecular mechanics and semi-empirical quantum chemical calculations has been applied (Sybyl software package). Data obtained from calculations have been stored and processed using Origin and Excel programs.

III. Results and conclusions

1, a) The SUAM-1221 has been studied by ^{1}H, ^{13}C, 2D NOESY, 2D TOCSY and also 2D HMBC and 2D HMQC NMR spectra recorded in different solvents.



trans *cis*

SUAM-1221

b) The assignment of *trans* and *cis* amide rotamers in solution has been executed mostly with the help of NOESY and ^{13}C NMR spectra. An interesting phenomenon has also been detected, namely that, in several cases, it is possible to differentiate between the amide rotamers on the basis of the ^{2}J proton-proton coupling constant of the prolyl δ (C(5)) protons. Through the assistance of the latter method the pyrrolidinyl 2'-H_2 and 5'-H_2 proton pairs have been identified. As far as we know this method has not yet been used for the identification of the proline amide rotamers in peptides.

c) Investigating the solvent effect on the *trans/cis* ratio it has been established that in apolar solvents the *trans* rotamer fully dominates, while in polar solvents this form is less favoured. The isomer ratios measured and obtained through molecular mechanics calculations differ considerably, while results of semi-empirical quantum chemical calculation show acceptable agreement with the experimental data.

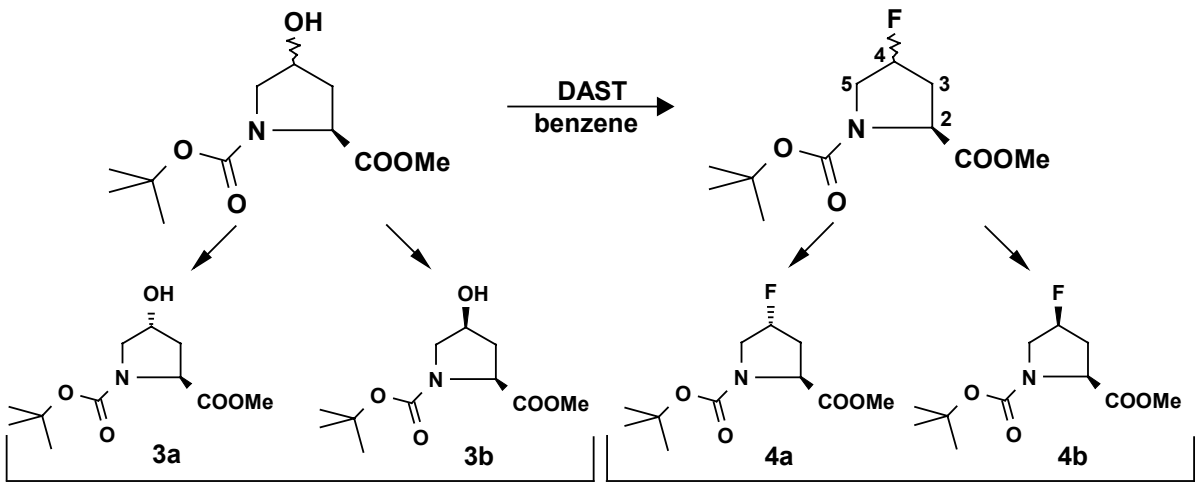
d) As the hindered rotation of the amide units of the prolyl- and pyrrolidinyl ring is a slow process on the NMR time scale, the rate constants of the *trans-cis* isomerization has been determined as well as the average lifetime of the *trans* and *cis* rotamers. It has been observed that shifting towards the more polar solvents the rate of the *trans-cis* rotation is getting slower.

e) It has been found for the prolyl ring that in the conformer set obtained through molecule mechanics calculations the "northern" (N), while according to the NMR data in solution the "southern" (S) conformer family dominates.

f) On the basis of the comparison of measured and calculated NOE integral values the relative orientation of the two five membered rings has been determined. The structure obtained in this way was in agreement with that obtained from molecular mechanics calculations.

g) The conformation of the aliphatic chain of SUAM-1221 (U-shaped/linear conformer ratio) has been attempted to determine via the application of the Haigh-Mallion equation parameterized by David A. Case. This method proved to be inappropriate for quantitative evaluation but on the basis of the measured and calculated data it has been assumed that the ratio of the U-shaped conformers in the solution was smaller than that calculated in vacuo.

2. Producing 4*α,β*-fluoroproline derivatives expectedly possessing PEP inhibitor effect via reacting substituted 4*α,β*-hydroxyprolines with diethylamino-sulfurtrifluorid (DAST) has been aimed. Besides producing new compounds it has also aimed to clear up the mechanism of the mentioned reaction.



a) The identification of the 4*α,β*-hydroxy- and 4*α,β*-fluoroproline derivatives has been accomplished on the basis of ^{1}H, ^{13}C, 2D NOESY, 1D gradient TOCSY, 2D TOCSY, and also 2D HMBC and 2D HMQC NMR spectra recorded in different solvents.

b) The assignment of *trans* and *cis* rotamers in solution has been performed with the help of NOESY and ^{13}C NMR spectra. It has been observed that in proline derivatives carrying an electronegative substituent in position four the *cis* and *trans* rotamers cannot be differentiated on the basis of the ^{1}H-^{1}H geminal coupling constant of the 5-H_2 protons. In all four compounds the NOE data and the coupling constants have confirmed the relative position of the hydroxyl and fluorine substituents.

c) Something interesting has been noticed through the use of the 1D gradient TOCSY method applied for the determination of ^{19}F-^{1}H spin-spin coupling constants in 4-fluoroproline derivatives, namely that by selective irradiating on the proton frequency one of

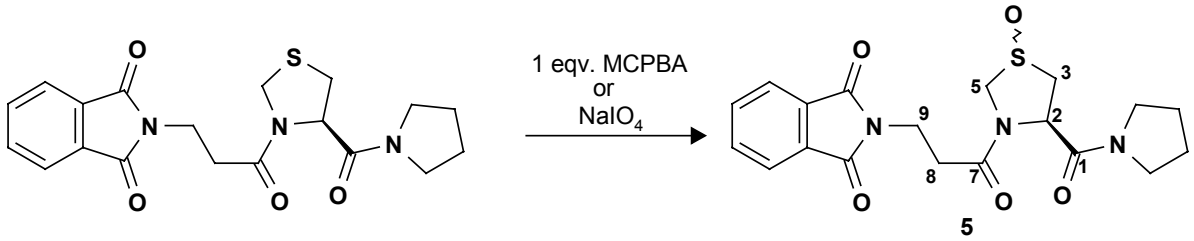
the component of the assigned doublet signal (4-H proton) of the *trans* rotamer we received a spectrum in which the signals appeared only on one end of the proton multiplet. The 1D gradient TOCSY experiment is analogous with the 2D HETLOC experiment; it is a one-dimensional version of that. The method detailed above can be useful when determining the spin-spin couplings in cases of overlapping signals of the 1D ^{1}H NMR spectrum; another advantage is the brevity of the experiment.

d) In accordance with literature data, in case of the 4*α*-hydroxy-, and the 4*α*-fluoroproline derivatives, the dominant conformation of the prolyl ring in solution is the N, while for the 4*β*-hydroxy- and 4*β*-fluoroproline derivatives the S conformation is the dominant one. Therefore, in solution, the conformation of the prolyl ring strongly depends on the position of the substituent connected to C(4) while the *trans* and *cis* rotamers of the same isomer have similar ring conformation.

e) It has been observed that the electronegative substituent in position four of the prolyl ring affects the rate of the *trans-cis* isomerisation; this is slower in the 4*α*-substituted derivatives than in case of the 4*β*-substituted compounds, supposedly because of steric effect. Comparison of the rate constants of *trans-cis* isomerisation of **3** (4*α*-) and **4** (4*β*-) isomers with that of SUAM-1221 indicates a smaller activation energy barrier for the former. This can be explained by the fact that the electronegative oxygen atom next to the amide carbonyl reduces the C-N double bond nature of the amide moiety.

f) These results provided explanation for the reaction between *N-tert*-butoxycarbonyl-4*α*- and *N-tert*-butoxycarbonyl-4*β*-hydroxyproline methylester and the DAST taking place through inversion similarly to examples described in the literature.

3. Starting from thioproline derivative 4-sulfoxide derivatives were obtained.



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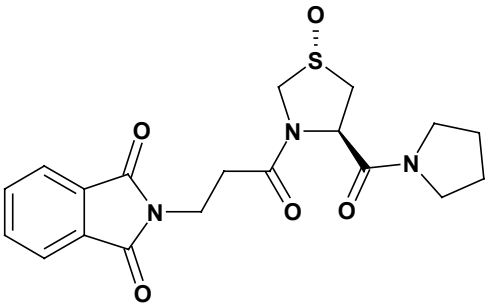
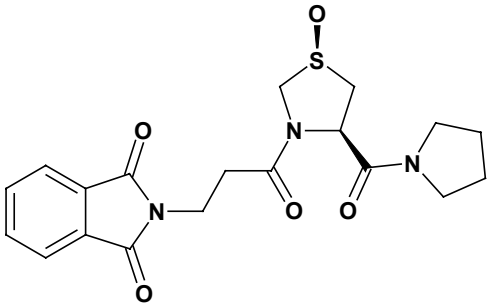
a) Four sets of signals have been detected in their ^{1}H, ^{13}C and 2D NOESY NMR spectra, which were assigned to *α* and *β* sulfoxide diastereomers, and to their *trans* and *cis* amide rotamers.

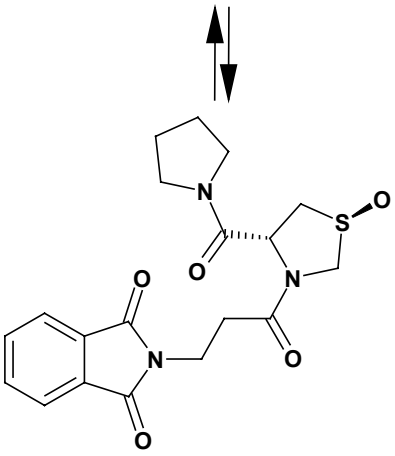
b) The obtained sulfoxide diastereomers have been differentiated with the application of the ASIS method. After definite assignments of the two sulfoxide isomers and the establishment of their configurations the ratio of *trans/cis* (major/minor) isomers has been determined with the help of HPLC. It has been observed that the stereoselectivity of the oxidation reaction remarkably depends on the reaction conditions. With MCPBA reagent practically only the *trans* (α-sulfoxide) isomer is produced (>99%), while the $NaIO_4$ results in a mixture of *trans* and *cis* isomers. It has been observed that an increase of reaction temperature led to a decrease of stereoselectivity.



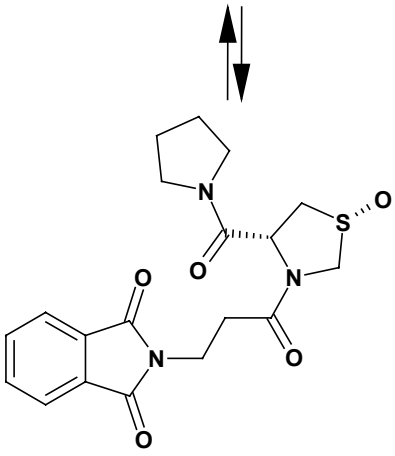
***trans* isomer - *trans* amide rotamer**



***cis* isomer - *trans* amide rotamer**

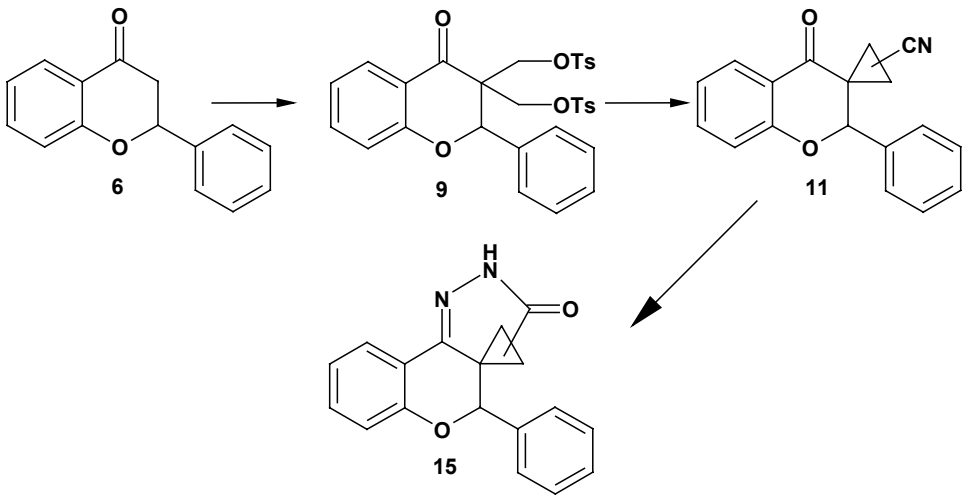


***trans* isomer - *cis* amide rotamer**



***cis* isomer - *cis* amide rotamer**

4. Starting from 2-phenyl-2,3-dihydro-4*H*-chromen-4-one (**6**) pyridazinone derivatives were obtained. The reaction of 3,3-bis-(tosyloxymethylen)-flavanone in the presence of cianide nucleophile was widely investigated.

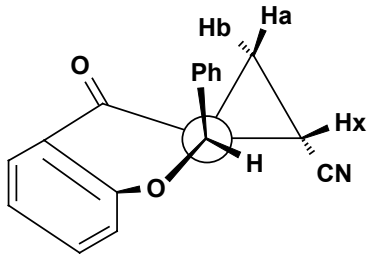


a) It has been established that, as opposed to other nucleophiles, here not a simple *SN* reaction takes place but, as a result of a SN_i reaction, three CN-substituted 3,3-spiro-cyclopropane-flavanone stereoisomers are formed.

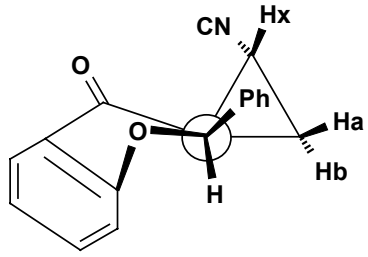
b) Methods for separating the stereoisomeric nitriles and for producing the appropriate amides and carbonic acids have been elaborated through hydrolysis. From the acids pyridazinon-spiro-cyclopropane-flavanone derivatives were obtained through reaction through the reaction with hydrazine hydrate.

c) The configuration of the 3,3 spiro-cyclopropane-flavanone stereoisomers has been established by applying different NMR techniques such as 1D ^{1}H-^{1}H NOE and two-dimensional 2D*J*-resolved NMR spectra. The $^{1}J_{C,H}$ spin-spin coupling constants have been determined from proton coupled ^{13}C NMR spectra.

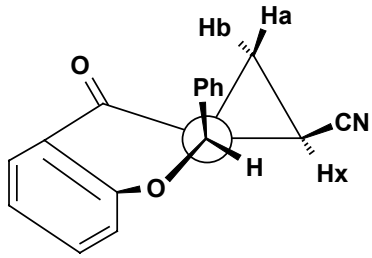
d) Finally, with the help of NMR measurements the configuration and conformation of three 3,3 spiro-cyclopropane-flavanone stereoisomers has been defined:



11A *(trans-cis* 2-$H_{ax/eq}$*)*

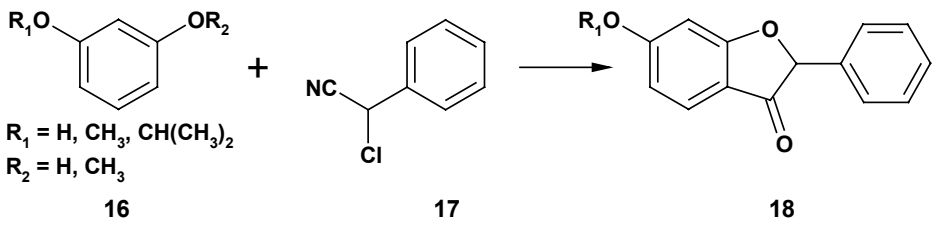


11K *(cis-cis* 2-H_{ax}*)*



11F *(trans-trans* 2-H_{eq}*)*

5. During the research on new materials affecting osteoporosis, the literature methods have been improved for the synthesis of a benzofuranone derivative.

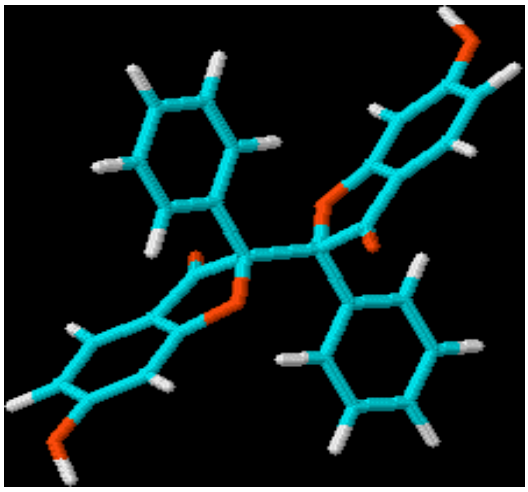


16 **17** **18**

a) The methods detailed in the literature have repeated and further improved, and also a proposal as for the mechanism of one of the reactions has made.

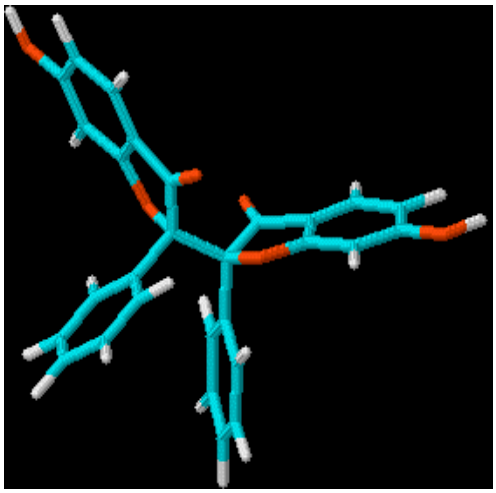
b) Besides the products reported, further molecules have been isolated and, with the help of ^{1}H, ^{13}C, DEPT-135, 1D gradient TOCSY, 1D selective INEPT, 2D HMBC, 2D HMQC, 2D NOESY NMR, and also IR and MS methods, their structures have unambiguously been elucidated.

c) Based on these results it can be concluded that the 3(2*H*)-benzofuranone and its derivatives are easily oxidized resulting in the formation of *meso* and *racemic* dimers as the main products.



meso

"R", "S" or "S", "R"



racemic (d,l-pair)

"R", "R" or "S", "S"

IV. Publications, oral presentations, posters

Publications involved in thesis:

1. Gy. Litkei, B.P. Khilya, A.B. Turov, L. Szilágyi, **S. Bokotey**, S. Antus:
"Synthesis of spirocyclopropyl acids from flavanones and their transformation to corresponding pyridazinone derivatives"
Khim. Get. Sojed., **5**., 616-623 (1995)

2. Benjamin Podányi, **Sándor Bokotey**, Károly Kánai, Miklós Fehér, István Hermecz:
"The investigation of a flexibile prolyl-endopeptidase inhibitor in solution by NMR techniques"
Magn. Res. Chem. **37,** 346-352 (1998)

3. Károly Kánai, Benjamin Podányi, **Sándor Bokotey**, Félix Hajdú and István Hermecz:
"Stereoselective sulfoxide formation from a thioproline derivative"
Tetrahedron Asymmetry, **13**, 491-495 (2002)

4. **Sándor Bokotey**, Mária Kővari-Rádkai, Benjamin Podányi, Imola Ritz, Zsolt Böcskei and Sándor Bátori:
"Studies on synthesis of 3(2*H*)-benzofuranone derivatives"
Synthetic Communications, in press

Other publications:

5. György M. Keserű, György T. Balogh, **Sándor Bokotey**, Géza Árvai, Béla Bertók:
"Metalloporphyrin catalysed biomimetic oxidation of aryl benzyl ethers. Implications for lignin peroxidase catalysis"
Tetrahedron, **55**, 4457-4466, (1999)

6. Márton Varga, Mária Kővári-Rádkai, Ildikó Prohászka-Német, Magdolna Vitányi-Morvai, Zsolt Böcskei, **Sándor Bokotey**, Kálmán Simon, István Hermecz and Sándor Bátori:
"Stability and chemical reactivity of 7-isopropoxyisoflavone (Ipriflavone)"
Eur.J.Org.Chem., 3911-3920, (2001)

Oral presentations and posters connected with thesis:

1. **Bokotey Sándor**, Kőváriné Rádkai Mária, Bátori Sándor, Podányi Benjamin, Ritz Imola, Böcskei Zsolt:
   "Structure elucidation of benzofuranone derivatives"
   Vegyészkonferencia, Eger, 1996. July 2-4.
2. **Bokotey Sándor**, Podányi Benjamin, Kánai Károly, Fehér Miklós, Hermecz István
   "The investigation of a flexibile prolyl-endopeptidase inhibitor in solution by NMR techniques"
   NMR Working Group of Hungarian Academy of Sciences, Budapest, SOTE 1997. May 28.
3. **Bokotey Sándor**, Podányi Benjamin, Kánai Károly, Fehér Miklós, Hermecz István
   "Conformational analysis of a prolyl-endopeptidase inhibitor"
   Vegyészkonferencia, Siófok, 1997. September 1-3.
4. Hermecz István, Kánai Károly, Susán Edit, Kapui Zoltán, Bátori Sándor, Erdő Sándor, Bence Judit, Fehér Miklós, Podányi Benjamin, Szeleczky Gábor, Böcskei Zsolt, Balogh Mária, Sipos Judit, Horváth Ágnes, Szappanos Andrea, Pappné Behr Ágnes, Szvobodáné Kanczel Ida, **Bokotey Sándor**, Bata Imre, Molnár Zsolt, Várkonyiné Schlovicskó Erika, Szatmári István, Simon Kálmán, Arányi Péter
   "Investigation of prolyl-endopeptidase inhibitors"
   Vegyészkonferencia, Siófok, 1997. September 1-3.
5. **Bokotey Sándor**, Podányi Benjamin, Horváth Ágnes, Hermecz István
   "Determination of the structure and conformational study of hydroxy and fluorine containing proline derivatives by NMR methods"
   XXI Kémiai Előadói Napok, Szeged, 1998. October 26-28.
6. **Sándor Bokotey**, Benjamin Podányi, Károly Kánai, István Hermecz
   "Determination of the configuration of sulfoxide moiety in thioproline derivative by NMR methods"
   Central European NMR Symposium and Bruker NMR Users Meeting, Szeged, 1999. September 2-3.

7. **Sándor Bokotey**, László Szilágyi, György Litkei, Sándor Antus
"Synthesis of 3-substituted flavanone derivatives and their structure elucidation by NMR methods"
MTA Flavonoidkémiai Munkabizottság tudományos előadó ülése, MTA Kémiai Kutatóközpont, Budapest, 2001.December 10.

Other oral presentations, posters and patents:

8. Simon A., Hajdú F., **Bokotey S.**, Ritz I.:
"Isolation of technological impurities and degradation products from Buformin HCl salt by HPLC method"
Elválasztástudományi Vándorgyülés'98, Lilafüred, 1998. September 30 – October 2.

9. "Synthesis of 1,5-disubstituted-3-amino-1,2,4-triazoles", Sanofi~Synthelabo, Paris, France.
Representative: Chinoin Pharmaceutical and Chemical Works Co. Ltd.
Inventors: **Bokotey Sándor**, Csikós Éva, dr Gönczi Csaba, Hajdú Félix, dr Héja Gergely, dr Hermecz István, dr Podányi Benjamin, Sántáné Csutor Andrea, Szomor Tiborné, Szvoboda Györgyné.
Registration number: P0004154, 2000. October 26.

10. "Chemical process and new intermediate", Sanofi~Synthelabo, Paris, France.
Representative: Chinoin Pharmaceutical and Chemical Works Co. Ltd.
Inventors: Sipos J., Szabó A., Horváth Á., Kiss Gy., Smelkó-Esek Á., Vasvári Á., Hermecz I., Nagy L.,Hajdú F., Simon A., Podányi B., **Bokotey S.**, Galambos G., Ivanics J.
Registration number: P0004741, 2000. November 28.