**MEDICAL UNIVERSITY – VARNA**

*"Prof. Dr. Paraskev Stoyanov"*

FACULTY OF PHARMACY

DEPARTMENT OF PHARMACEUTICAL CHEMISTRY

# *„Synthesis, structure and properties of new iodine derivatives of natural aromatic acids"*

Extended abstract of a PhD thesis

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The dissertation was discussed at a meeting of the Department Council of the Department of Pharmaceutical Chemistry at the Medical University - Varna "Prof. Dr. Paraskev Stoyanov" and is aimed at defense before the scientific Jury.

The dissertation covers 113 pages, 61 figures, 14 tables and 16 diagrams.

The dissertation includes 6 applications. 148 titles are cited.

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The public defense of the dissertation will take place on..... year from ..... in the hall ..... outdoors meeting of the scientific jury.

## Introduction.

During the last few decades, halogens have played an important role in the development of pharmaceuticals. It is a well-known phenomenon that both the effectiveness and metabolic stability of many drug molecules can be significantly enhanced by inserting halogen atoms into their compositions. The potential of halogens to improve the oral absorption of several drugs has also been shown. Halogenation improves the main pharmacokinetic and pharmacodynamics parameters of medicinal substances. It is no coincidence that ¼ of the total number of scientific papers and patents in the field of medicinal chemistry concerns the synthesis of new halogen-substituted compounds. Halogen-substituted organic compounds are also identified as important precursors in organic synthesis. Currently, a significant number of newly developed drug molecules contain a halogen atom. Halogenation is established as one of the most valuable approaches in the development of new molecules. Strategies for introducing halogen atoms into the carbon 'backbone' of new and known medical substances are the most diverse and cover different groups of natural and synthetic compounds, such as nucleosides, alkaloids, macrolides, steroids, amino acids, prostaglandins, and benzodiazepines. Far from being a coincidence, halogenated heterocyclic and aromatic residues are present in all pharmacopeias. It has been proven that the presence of only one halogen atom can convert almost any biologically indifferent molecule to a pharmacologically active one. The halogen atom is isosteric and isopolar to many organic functionalities. The synthesis of new and much more effective polyhalogen-substituted anesthetics and opioids is currently being actively pursued. Over the last 5 years, there has also been a growing interest in the isolation of new natural halogen-containing compounds. Organoiodic compounds are important industrial products and intermediates for the synthesis of several drugs and synthetic polymers. More than 2,000 iodine-containing compounds have been identified as naturally occurring compounds produced by many plants, fungi, bacteria and marine organisms. The importance of organohalides is growing rapidly. It is not by chance that 7 of the best-selling top 10 drugs in the United States in recent years are halogenated compounds, and 80% of newly developed agrochemicals contain one or more halogen atoms in their compositions. Therefore, the introduction of halogen atoms in organic molecules is one of the most important and targeted transformations in modern organic and pharmaceutical synthesis[1].

From the prepared literature review, it should be said that the reactions of halogenation (iodination) of organic (including medicinal) substrates are one of the most significant chemical transformations in pharmaceutical synthesis. No less important are the reactions that use the already obtained iodine-substituted compounds for the synthesis of much more complex ones.

In addition, because of the apparent deficiency of iodine-containing contrast agents, the synthesis of new organoiodic compounds is extremely valuable and significant.

## II. AIM OF THE RESEARCH STUDY

Set goals:

To synthesize and study the structural features and some of the biological properties of a series of new organoiodic substances and evaluate their potential in the synthesis of other substances.

The following tasks were set for the realization of the set goals:

1. To synthesize three- and tetraiodosubstituted aromatic acids, derivatives of the radiopaque Amidotrizoic acid.
2. Investigate the effectiveness of various iodinating agents for the synthesis of the target 2,6-diiodo-3,4,5-trimethoxybenzoic acid.
3. Structurally characterize the newly synthesized DITMBA using FTIR and NMR spectral analysis.
4. Evaluate the potential cytotoxic and genotoxic effects of DITMBA.
5. Determination of the activity of DITMBA against *Gram* - (-) and *Gram* - (+) bacteria and fungi, representatives of the *Candida species*.
6. Carried out the synthesis of *meta*-terphenyl derivatives of some of the obtained diiodosubstituted acids.
7. Synthesize organic crystals with DITMBA.

## III. MATERIALS AND METHODS

### 1. MATERIALS

3,4,5-trimethoxybenzoic acid (99 +%, Alfa Aesar), 3,4-dimethoxybenzoic acid (99 +%, Acros Organics), 3,5-dimethoxybenzoic acid (99%, Acros Organics), trifluoroacetic acid (99 %, Alfa Aesar), iodine (≥ 99.8%, Sigma-Aldrich), silver nitrate (> 99.5%, Fluka), sodium hydroxide (BioXtra, ≥98%, Sigma-Aldrich), chloroform (anhydrous, contains amylenes as a stabilizer, ≥99%, Sigma-Aldrich), cyclohexane (anhydrous 99.5%, Sigma-Aldrich), toluene (anhydrous 99.8%, Sigma-Aldrich) and dichloromethane (anhydrous, ≥99.8%, contains 40-150 ppm amylene as a stabilizer, Sigma- Aldrich), hydrogen peroxide (50 wt.% In H2O, stabilized, Fluka), methanol (99.9%, extra dry, AcroSeal®), ethanol (absolute, reag. ISO, reag. Ph. Eur., ≥99.8%, Sigma -Aldrich), potassium metabisulfite (98%, Fluka), sulfuric acid (ACS reagent, 95.0-98.0%, Fluka), 45% acetic acid (≥99.99%, Sigma-Aldrich), ethanol (absolute, ≥99.8%, Sigma -Aldrich) and 1% solution of acetoorcein (Sigma-Aldrich).

### 2. METHODS

**2.1 The IR spectra** was recorded on an infrared spectrophotometer (FT-IR) models Bruker Tensor 27 and Bruker Tensor II, equipped with ATR attachment. Samples were taken using 64 scans, in the form of CsI tablets or in their "native" state (in cases where the spectra were taken using an ATR attachment).

**2.2 The $^{1}$H NMR and $^{13}$C NMR spectra** of the target compound were recorded on a Bruker Avance DRX 250 spectrometer at 250 MHz using deuterated chloroform as a solvent and tetramethylsilane as an internal standard.

**2.3 The melting point** was determined on an A. Krüss KSP1N melting point apparatus.

**2.4 The QCM (Quartz Crystal Microbalance) method** was used to determine the total iodine content of the organoiodic compounds obtained. The QCM-based thermogravimetric method is based on measuring changes in the frequency of the quartz membrane as the mass of a sample applied to it changes. The piezoelectric quartz plate used for this purpose is 200 μm thick and 14 mm in diameter, obtained from a quartz single crystal. The use of this method in the present

thermogravimetric study is associated with the use of original adsorption QCM equipment.

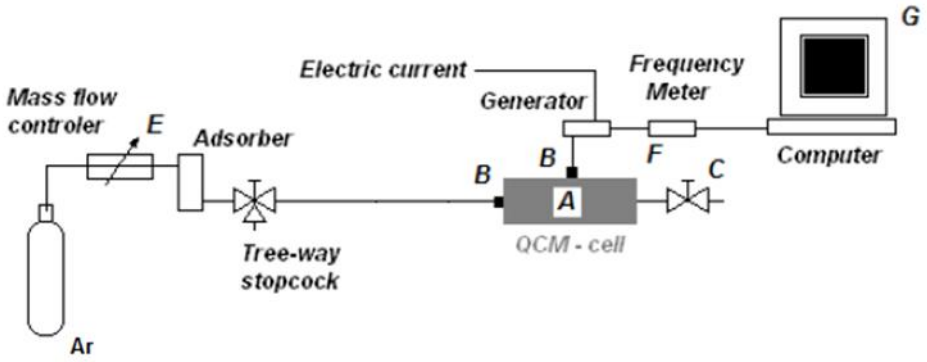



Figure 1. Experimental QCM setup for the assessment of iodine content in organoiodines

The organoiodine compounds synthesized by us are applied to the surface of the Au electrode of the quartz plate, in the form of a toluene solution, using a micropipette. The sample placed in the chamber is activated in a stream of "dry" Ar. After establishing a constant signal, the camera is heated in a stream of warm air generated by HotGun (Steinel HG 2310 LCD). Fine adjustment of the carrier gas is achieved by means of a flow regulator (E). The change in the operating frequency of the QCM sensor is determined using the BK precision 1823A (F) frequency meter. The change in the mass of the QCM sensor, respectively the change in frequency, is recorded every second using a personal computer equipped with specialized software (G). The reported changes in the frequency of the quartz analyzer are to its traceable, ionic state. The *Volgard* analysis was performed in a strongly acidic medium to prevent hydrolysis of ferric iron, which resulted in undesirable staining of the analytical solution.

Approximately 0.05 g of reaction product was weighed into exactly 15 mL of a pear-shaped flask with a slice. To this was added 1.7 mL of NaOH solution (15%), 7.0 mL of bi-distilled water and 0.34 g of zinc powder. The resulting mixture was boiled for 1.5 hours. The condenser used was carefully washed several times with

bi-distilled water (in 1.0 mL portions each). All washes were combined with the reaction solution and transferred quantitatively to a 25 mL volumetric flask. To 10 mL of the resulting solution was added 2.5 mL of 0.1 N silver nitrate solution and 2.5 mL of dilute (1: 1 v / v) $HNO_3$ solution. Excess $AgNO_3$ was titrated with 0.1 N ammonium thiocyanate solution. 0.2 mL of saturated alum solution was used as an indicator. Titration was performed using a 2.0 mL graduated microburette (Herka® Intercolor). The titrations were repeated three times. The results are expressed in molar equivalents.

recalculated as changes in the mass of the analyzed organoiodic agent using the Saurbery equation:

$$\Delta m=(\rho NA/f^2)\Delta f$$

**2.5 The *Volhard* titrimetric method** was used to determine the total iodine content of the newly synthesized compounds. The *Volgard* method uses the technique of inverse argentometric titration. Argentometric titration by the *Volgard* method is performed after pre-conversion of the covalently bound iodine

**2.6 The high-performance liquid chromatography (HPLC) analysis** was performed using a Thermo Scientific UltiMate 3000 Analytical LC System equipped with a quaternary pump (Thermo Scientific Dionex UltiMate 3000 LPG-3400SD Quaternary Pump), an automatic injector (Thermo Scientific Dionex UltiMate 3000 Autosampler) and a diode matrix detector (Thermo Scientific Dionex UltiMate 3000 VWD-3100 Variable Wavelength Detector/VWD). System control, data collection and analysis were performed using Thermo Scientific™ Chromeleon™ 7.2 Chromatography Data System software. Chromatographic analysis was performed using a mobile phase fed at a rate of 1.0 mL/min. The separation was performed using a Hypersil GOLD aQ C18 chromatographic column (150 mm x 4.6 mm, 5 μm, Thermo Scientific USA, USA), protected by a pre-column Hypersil GOLD aQ C18 (10 mm x 4.0 mm, 5 μm, Thermo Scientific ™, USA).

The chromatographic column and pre-column were thermostated at 30 ° C and the autosampler at 10 ° C. Samples were injected in a volume of 20.0 μL each. A photodiode detector is used for this purpose. The analysis was performed at the following operating wavelengths - 295, 290, 285, 280, 275, 270, 265, 260 nm.

The analysis was performed in gradient mode. The total duration of the analysis was 30.0 minutes.

**2.7 X-ray examinations** were performed with a Planmeca ProMax 2D apparatus (Planmeca, Helsinki, Finland), integrated with a computer, archiving peripherals and specialized imaging software. All set operating parameters are presented in Figure 2.

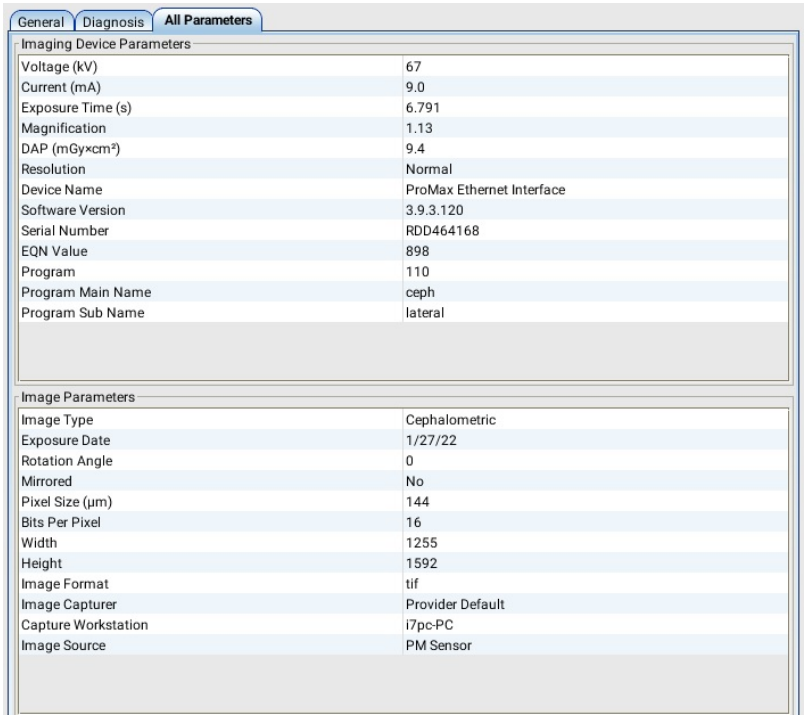



Figure 2. Mainly set X-ray parameters

The iodine-containing solutions tested have an equivalent iodine content of 370 mg / mL $I_2$. All test solutions, with a volume of approximately 50 µL, were placed in borosilicate capillaries. The latter are placed at a distance of 1 cm from each other in a styrofoam holder.

### 2.8 Powder X-ray diffraction analysis (PXRD)

The radiographs were taken using a Philips PW1710 X-ray diffractometer equipped with a Cu Kα anode emitter. Data were collected at room temperature and at an angle of reflection (2θ) in the range of 5 to 50 °.

## 3. Synthetic methods

**3.1 Synthesis of silver (I) oxide** by the following reaction route:

$$2\ AgNO_3 + 2\ NaOH \longrightarrow Ag_2O + 2\ NaNO_3 + H_2O$$

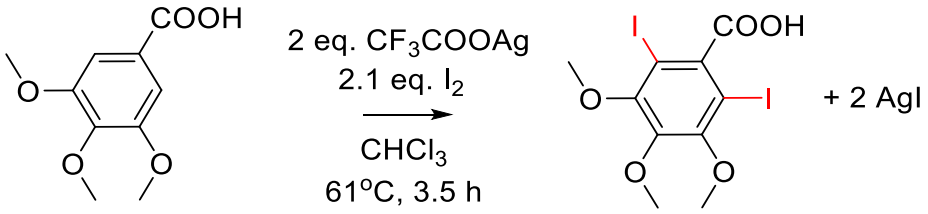
Silver oxide is obtained by adding, with constant stirring, an aqueous solution of NaOH with conc. 5.0 g / 100 mL, to aqueous $AgNO_3$ with conc. 20g / 100mL (0.118 mol). The precipitate formed is collected by filtration and washed with water until neutral. The precipitate is used in „crude“ form for the synthesis of silver trifluoroacetate.

**3.2 Synthesis of silver trifluoroacetate** by the following reaction route:

$$Ag_2O + 2\ CF_3COOH \longrightarrow 2\ CF_3COOAg + H_2O$$

To a suspension containing 5.0 g (0.0215 mol) of silver oxide in 200 mL of water was added 4.92 g (3.3 mL, 0.0431 mol) of trifluoroacetic acid. The resulting solution was filtered and the filtrate was evaporated to dryness. The resulting silver trifluoroacetate can be purified in two ways: by extraction with a Soxhlet apparatus or by filtration through a thin layer of activated carbon with diethyl ether. A thin layer activated carbon filtration approach is applied here. The yield of silver trifluoroacetate was 8.7 g (91.3%).

**3.3 Synthesis of 2,6-diiodo-3,4,5-trimethoxybenzoic acid with $I_2$ / $CF_3CO_2Ag$ iodinating reagent:**



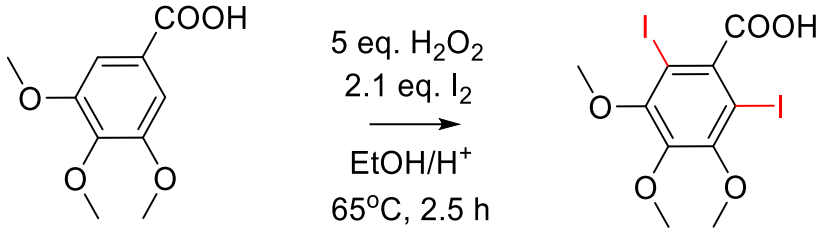

A 250 mL round bottom flask equipped with a magnetic stirrer, a reflux condenser and a two-way adapter was charged with 1.5 g (7.07 mmol) of 3,4,5-trimethoxybenzoic acid and 3,123 g (14.14 mmol) (freshly prepared) silver trifluoroacetate. At an earlier stage, the reaction system is subjected to repeated "evacuation" under vacuum and filled with "dry" argon, using a double collector system – argon/vacuum.

1.83 g (7.20 mmol or 1,018 equivalents) of iodine and 15 mL of chloroform were placed into the dropping funnel under an argon atmosphere. Heat the reaction flask to 65 ° C in an oil bath. When the temperature stabilizes, the magnetic stirrer is turned on, and after a few minutes the controlled slow addition of iodine to the

reaction mixture is ensured; in our case, the continuous introduction of iodine into the reaction volume takes place within 2 hours.

After the final addition of all the iodine, the reaction mixture was refluxed for 2.5 hours and then cooled to room temperature. The silver iodine precipitate was removed by filtration and washed several times with 5.0 mL of chloroform. The resulting filtrate was concentrated to dryness under reduced pressure.

### 3.4 Synthesis of 2,6-diiodo-3,4,5-trimethoxybenzoic acid with $I_2$ / $H_2O_2$ iodinating reagent:




50 mL of absolute ethanol placed in a 200 mL round bottom flask equipped with a magnetic stirrer, reflux condenser and dropping funnel. Heat the ethanol in a water bath to 65 ° C and add to it the iodine - 1.83 g (7.20 mmol, 1,018 equivalents). The solution was stirred until the iodine crystals were completely dissolved and then cooled to room temperature. To the solution thus obtained was added 1.5 g (7.07 mmol) of 3,4,5-trimethoxybenzoic acid and 5.0 mL of sulfuric acid. Using a dropping funnel, add 15.0 mL of hydrogen peroxide at a rate of 3 to 4 drops per minute. The reaction temperature must not exceed 15 °C; periodic ice cooling is applied. After adding all the hydrogen peroxide, the reaction system stand for 20 minutes and then added slowly, in portions and with constant stirring, to a pre-prepared and cooled 4% aqueous solution of potassium metabisulphite (600 mL). The crystalline precipitate formed is filtered off, washed with potassium metabisulphite solution and dried at room temperature in a place protected from direct sunlight and then in a desiccator.

### 3.5 Synthesis of 2,6-diiodo-3,4,5-trimethoxybenzoic acid (DITMBA) involving $I_2$ / $AgNO_3$ iodinating reagent.

A 50 mL round bottom flask equipped with a magnetic stirrer was charged with 1.44 g of 3,4,5-trimethoxybenzoic acid (6.786 mmol), 3.87 g of I2 (2.25 eq; 15.25). mmol), 2.58 g silver nitrate (2.25 eq; 15.17 mmol) and 25 mL methanol. The contents of the container are protected from light by Al film. The reaction mixture was stirred at room temperature for 24 hours under a stream of argon (0.5 mL/min). The resulting red-brown filtrate was gradually transferred to a 0.5 liter beaker equipped with a magnetic stirrer, 0.5 g $NaHSO_3$ and 300 mL water. The desired product, separated as a white amorphous solid, was collected by suction filtration and washed three times with a little water.

### 3.6 Synthesis of triiodosubstituted aromatic acids with the participation of $I_2$ / $AgNO_3$ iodinating reagent.

All reactions were performed under normal conditions, using standard 50 mL flat-bottom flasks. 3.1 equivalents of $AgNO_3$ (4.34 g, 25.525 mmol) and 3.1 equivalents of $I_2$ (6.48 g, 25.525 mmol) were used to iodize the two aromatic acids (1.5 g, 8.234 mmol). Dissolve the contents of each flask in 30 mL of anhydrous methanol. The reaction mixtures were stirred on a magnetic stirrer at room temperature (21 ° C) for 24 hours. The flasks are pre-covered with aluminum foil to protect them from light. The mixtures were then filtered and the resulting filtrates were concentrated in a vacuum at 90 ° C.

### 3.7 Synthesis of tetraiodosubstituted aromatic acids with the participation of $I_2$ / $AgNO_3$ iodinating reagent.

For the iodination of 3-methoxybenzoic (3-MBA) and 4-methoxybenzoic (4-MBA) acid (1.0 g, 6.572 mmol) 4.1 equivalents of $AgNO_3$ (4.58 g, 26.945 mmol) and 4,1 equivalent of $I_2$ (6.84 g, 26.945 mmol). All substances, for each experiment, were charged into standard 50 mL single-necked flasks. Dissolve the contents of the flasks in 30 mL of anhydrous methanol. The reaction mixtures were stirred on a magnetic stirrer at room temperature (21°C) for 24 hours. The flasks are covered with aluminum foil to protect against the daylight and artificial light. The mixtures were then filtered and the resulting filtrates were concentrated in vacuo at 90°C for 1 hour. The obtained crude products were subjected to

spectral (FTIR and NMR) and titrimetric analysis. All syntheses were repeated three times to confirm the repeatability of the synthetic method.

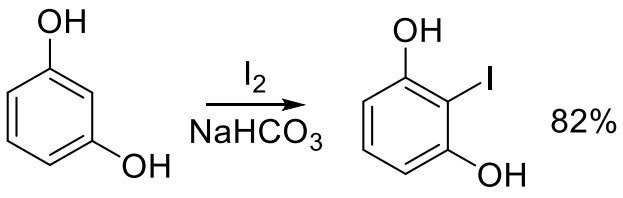
### 3.8 Synthesis of 2-iodobenzoic acid.

2-Aminobenzoic (anthranilic) acid (10.0 mmol, 1.00 eq.) was added to a pre-cooled to 0 ° C solution of $H_2SO_4$ (1:2 v/v) in a volume of 15 mL. Methanol (approximately 10 mL) was added to the contents of the flask until the introduced anthranilic acid was completely dissolved. Over a period of approximately 30 minutes, at 0 ÷ 5 ° C, a pre-prepared aqueous $NaNO_2$ solution (759 mg, 1.10 eq.) was slowly added dropwise to the solution thus obtained. The contents of the flask were stirred with a magnetic stirrer for 15 minutes. 8.3 g of KI (5.00 eq.) are then added. The contents of the flask were heated at 60°C for 1 hour. The product was washed with 50 mL of water and an equivalent amount of 10% $Na_2S_2O_3$ solution. Recrystallized from the water-methanol mixture. The resulting product was dried in a vacuum desiccator over $P_2O_5$. The sample is stored at low temperature in the absence of light.

### 3.9 Synthesis of biphenyl and *meta*-terphenyl derivatives.

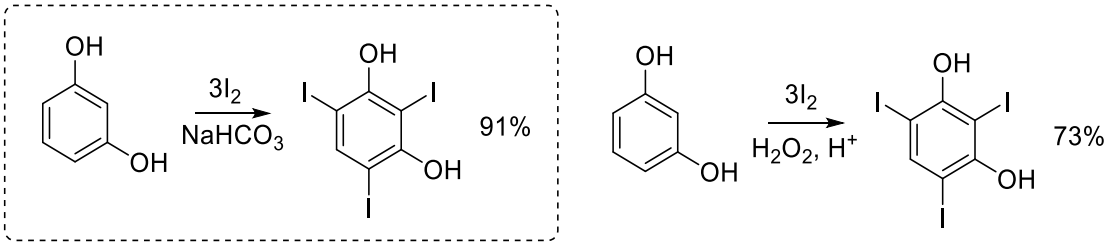
$K_2CO_3$ (0.212 g, 2 mmol), Pd $(OAc)_2$ (2 mg, 1 mol%), PEG 4000 (2.0 g) and bidistilled water (3 mL) were charged to a 50 mL single neck flask. The contents of the flask were heated (50°C) and stirred with a magnetic stirrer for 15 minutes. To the homogeneous mixture thus obtained was added, in one portion, phenylboronic acid (or 4-methoxyphenylboronic acid) and the iodine-substituted aromatic acids used. (2-iodobenzoic acid and DITMBA). The reaction time is 5 hours**.**

### 3.10 Synthesis of 2-iodoresorcinol (2-iodobenzene-1,3-diol):



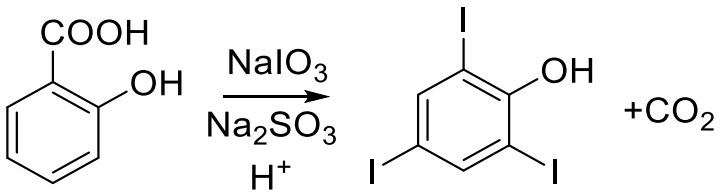
Distilled water (20 mL), resorcinol (2.75 g) and iodine (6.7 g) were placed in a one-necked round bottom flask equipped with a magnetic stirrer. Cool the flask with an ice water bath and slowly add $NaHCO_3$ (2.3 g) to its contents; divided into equal, small portions. During the reaction, the release of carbon dioxide is observed. After adding the entire amount of $NaHCO_3$, the reaction medium is allowed to react for 15 minutes and then warmed to room temperature. The solution was stirred for another 10 minutes, during which time a brown precipitate formed. The product was extracted with ethyl acetate (3 x 50 mL), then washed with diethyl ether and dried over $MgSO_4$. The product obtained (2-iodoresorcinol) is a creamy solid. T.t. 107-109°C. (lit. 106-110$^0$C).

### 3.11 Synthesis of riodoxol (2,4,6-triiodobenzene-1,3-diol):




To 0.55 g of resorcinol, 3.8 g of iodine and 20 mL of water was added with constant stirring and in small portions 1.3 g of $NaHCO_3$; the temperature of the reaction mixture is monitored not to exceed the threshold of 25°C. The resulting product was extracted with chloroform (3 × 6.0 mL), then filtered and dried at 50°C to constant weight. T.t. 152$^0$C (lit. 154$^0$C).

### 3.12 Synthesis of 2,4,6-triiodophenol (2,4,6-triiodophenol):



In a beaker equipped with a magnetic stirrer, dissolve 1.38 g (0.01 mol) of salicylic acid, 1.98 g of sodium iodate (0.01 mol) and 2.52 g of sodium sulfite (0.02 mol) in a mixture of 5.0 mL of methanol and 40.0 mL of distilled water. To the solution thus obtained, 0.01 mol of hydrochloric acid was added with constant stirring over 2.5 hours. A suspension is obtained which is filtered and washed with water. Free iodine is removed by adding a 5% sodium sulfite solution. Yield: 70%. T.t. $160^0$C (lit. 157-$159^0$C).

### 3.13 Synthesis of cocrystals involving 2,6-diiodo-3,4,5-trimethoxybenzoic acid.

25mg of 2,6-diiodo-3,4,5-trimethoxybenzoic acid and an equimolar amount of drug substance (Nitrofural, Metronidazole, Nitrofurantoin, Nifuroxazide) were charged to a crystallizer. The compounds were dissolved in 10 mL of ethanol. The resulting solution was heated for about 2 minutes in a water bath at 60°C until the dissolved substances were completely dissolved. The crystallizer is left at room temperature to slowly evaporate the solvent.

### 3.14 Synthesis of N-(1-(3,4-dihydroxy-5-methyltetrahydrofuran-2-yl)-5-fluoro-2-oxo-1,2-dihydropyrimidin-4-yl)-2,6-diiodo-3,4,5-trimethoxybenzamide.

0.25 g of DITMBA (5.39 mmol), 0.132 g of 5'-deoxy-5-fluorocytidine (5.39 mmol) and 0.111 g of N, N'-dicyclohexylcarbodiimide (5.39 mmol) were charged to a 50 mL round neck flask equipped with a magnetic stirrer. The contents were dissolved in 30 mL of dichloromethane. The reaction mixture was stirred on a magnetic stirrer at room temperature (21°C) for 48 hours. The flask is pre-covered with aluminum foil. The reaction solution was then filtered and the resulting filtrate was concentrated in a vacuum to dry. The expected product is separated by column chromatography.

## 9. Biological methods.

9.1Evaluation of the cyto- and genotoxic effects of DITMBA.

9.1.1 Obtaining roots from *Allium cepa L.*

The bulbs of the plant *Allium cepa L.*, purchased from a certified organic producer, were used as a test object. The bulbs, cleaned of yellow scales and dried roots, were placed in beaker cups in distilled water at a temperature of 25°C. Incubation of the bulbs lasts 24 hours; to the formation of roots with a length of about 1.0 cm.

9.1.2 Preparation of working (test) solutions.

Test solutions were prepared immediately before each analysis. For this purpose, solutions of 5-Fluorouracil at a concentration of 0.1 mg / mL and of DITMBA at concentrations of 0.3 and 0.16 mg / mL were prepared. The bulbs (their roots) were placed in the test solutions thus prepared for a period of 24 hours at a temperature of 25 ± 1°C. Distilled water was used for the negative control. After the treatment step, the bulbs and roots are washed thoroughly with distilled water. The roots were cut and fixed with Clark's fixative containing 96% ethyl alcohol and glacial acetic acid (3:1 ratio). The fixation process lasts 90 minutes at constant temperature (25°C). The following is a procedure of washing the roots with 96% ethyl alcohol. The samples were stored in 70% ethanol at 4°C.

9.1.3 Preparation of microscopic slides by the *Fiskesjo* method.

It works with a standard aceto-orcein mixture (a mixture of 3 parts ethanol and 1 part glacial acetic acid), in which the roots are fixed and placed in the refrigerator for 24 hours. The roots were fixed in 1 N HCl for 3 minutes and transferred again to the aceto-orcein solution for 30 minutes. The tips of the roots were removed to 0.75 mm with a scalpel and washed with 45% acetic acid for 10 seconds. Place the samples on a glass slide and add two drops of 45% acetic acid. A cover glass is placed over them, which is pressed. The windows are fixed with colorless varnish. The preparations were observed under a light microscope at a magnification of 400X.

9.1.4 Analysis of temporary microscopic preparations.

Observations of cell nuclei were achieved with a binocular microscope Model BM-180 / SP, equipped with a digital 3.14 MP camera.

9.2 Analysis of antimicrobial activity

9.2.1 Determination of minimum inhibitory concentrations (MICs) of chemicals by the serial dilution method.

Autoclaved pieces of paper measuring approximately 10 $mm^2$ are loaded with different amounts of test substances and placed in sterile tubes. To each tube was

added 1.0 mL of mesopepton broth (MPB broth) and 0.1 mL of bacterial suspension (*Staphylococcus aureus*, *Escherichia coli* or *Candida albicans*) at a density of 5 x 105 cfu / mL. The tubes were inoculated at 36 ± 1°C for 24 hours

The MIC determination was taken using *Mueller Hinton* Broth liquid medium (M-H Broth), sterile filter paper, sterile tubes and autoclavable distilled water. The turbidity of the tested cell suspensions was determined using a table densitometer model DEN-1; Calibrated to measure turbidity from 0.00 to 6.00 *Mc Farland* units.

9.2.2 Determination of minimum inhibitory concentrations (MICs) of chemical agents by the classical dilution method.

All organoiodic compounds were pre-dissolved under sterile conditions and at well-defined concentrations in Mueller Hinton broth. Place 2.0 mL of each test substance in tube №1. 1.0 mL of broth was dispensed into five tubes numbered 2 to 6, respectively. Tube №1 was homogenized and 1.0 mL of its contents was withdrawn and transferred to tube №2. This was repeated sequentially to tube №6, from which 1.0 mL was withdrawn and removed. The positive control is a tube that contains - broth + inoculum. 1.0 mL of diluted, standardized inoculum was added to all tubes. The tubes were incubated at 37°C for 24 hours. MIC is recorded as the lowest concentration of analytes (highest dilution) that completely inhibits bacterial growth.

## IV. RESULTS AND DISCUSSION

### 1.Synthesis of 2,6-diiodo-3,4,5-trimethoxybenzoic acid (DITMBA).

The reaction pair iodine-silver trifluoroacetate $I_2$/$AgOOCCF_3$ was used for the complete iodination of 3,4,5-trimethoxybenzoic acid to the product DITMBA. Spectral analysis of the reaction product showed that the use of this reagent provided us the maximum yield (98%) and with the maximum purity of the desired diiode-substituted product (≥ 99%). The DITMBA obtained with reagent pair $I_2$ / $AgOOCCF_3$ was used for the subsequent spectral analysis, as well as as a precursor for the synthesis of *meta*-terphenyl derivatives, cytidine-containing amide and organic cocrystals.

### 2. FTIR analysis of DITMBA[2]

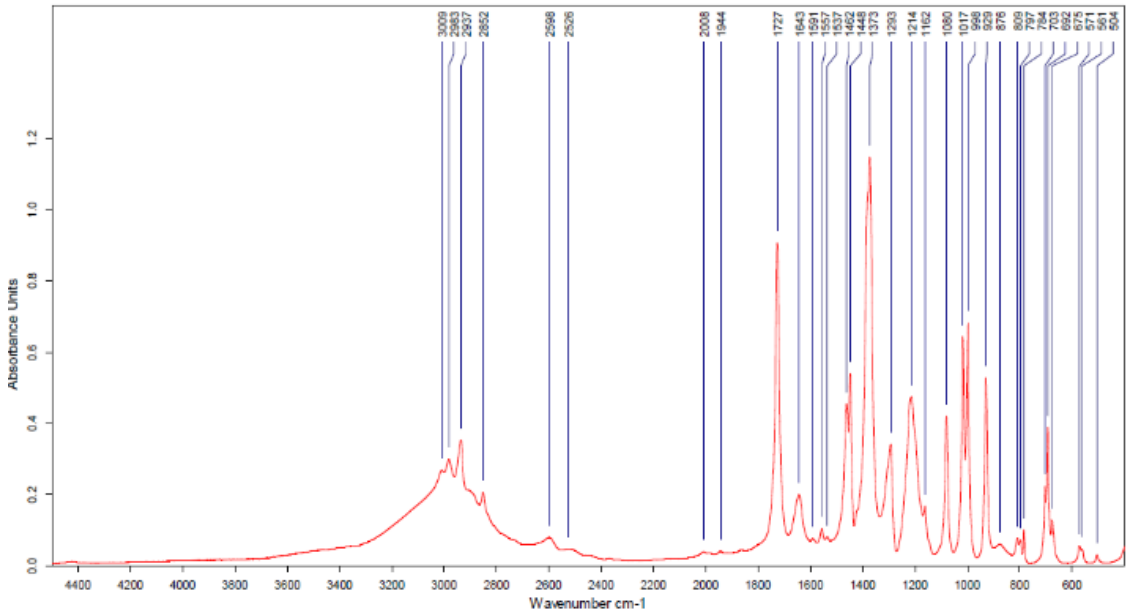



Figure 3. FTIR spectrum of 2,6-diiodo-3,4,5-trimethoxybenzoic acid

The vibrational frequencies of DITMBA were determined by comparative analysis of the data from its IR spectrum (Fig. 3) with those of ITMBA, studied earlier in [3]. In this way, we can observe important spectral changes after the replacement of the hydrogen atom with iodine. As can be expected, this structural transformation mainly affects the vibrations of the benzene ring, the C-I, the carboxyl group and to a lesser extent the vibrations of the $H_3C$-O groups.

A significant change in the molecular force field is observed vibrations of the benzene ring, which usually fall in the range of 1600 ÷ 1450 $cm^{-1}$. Here we found three bands that, unlike the ITMBA, are low intensity and shifted in their frequency position.

Unlike the spectral data of ITMBA IR, where a single C-I band is observed at 595 $cm^{-1}$, in the DITMBA IR spectrum, we observe a doublet of two valence C-I (asymmetric and symmetric) vibrations at 571 and 562 $cm^{-1}$.

In the crystalline phase, each pair of DITMBA molecules associate in carboxyl dimers, similar to ITMBA and other aromatic acids. In the IR spectrum of DITMBA, the characteristic bands for ν (C = O) oscillation are observed at frequencies 1644 and 1726 $cm^{-1}$

Following the above scheme for comparing the spectral characteristics of ITMBA and DITMBA, we found that there is no significant difference in the position of

the symmetric and asymmetric valence C-H bands characteristic of the methoxy groups.

2.1 Features and specific spectral behavior of DITMBA dimer.

In the crystalline phase, each pair of DITMBA molecules associated in carboxyl dimers, similar to ITMBA and other aromatic acids. In the IR spectrum of DITMBA, the characteristic bands for ν (C = O) oscillation are observed at frequencies 1644 and 1726 $cm^{-1}$.

The unusual spectral behavior of a DITMBA dimer can be represented as a superposition of two effects: on the one hand, it is the steric effect (due to diode substitution in the ortho position) that "pushes" the carboxyl dimer plane out of the plane of benzene rings, thus the steric effect causes an increase in the frequency of both the valence ν (C = O) oscillation and those of the out-of-plane γ (-OH ... O) oscillations. On the other hand, there is a strong polarization effect between C-I dipoles with those of carboxyl functional groups. The electric field created by them affects the intensity and position of the individual components of the carbonyl band. This in addition explains the increase in the intensity of the γ (-O-H ... O) band. The structural changes required by the ortho effect are expressed by the change in the angle α between the planes of the carboxyl dimer and the aromatic rings in these three compounds.

The valence O-H vibrations of the carboxyl dimer should be sufficiently sensitive to these electro-structural effects. Unfortunately, however, the profile of O – H spectral bands is extremely complex in the solid state. In this case, the full set of valence O – H oscillations is represented by a significantly wide band in the range $3200 \div 2500\ cm^{-1}$. The overlap of this broad O – H absorption band (as with the ITMBA) in the presence of C – H bands (of –OCH3 groups) makes it difficult to assign the valence bands of the O – H group at 3146 and 3071 $cm^{-1}$. However, the characteristic off-plane γ (-O-H ... O) oscillation of the acid dimer turned out to be much more interesting and indicative of its strength. The observed high-frequency shift of this band in the DITMBA spectrum (at 928 $cm^{-1}$) compared to that in the ITMBA spectrum (at 918 $cm^{-1}$) is accompanied by a significant increase in the intensity of the former. The lack of coupling between the DITMBA acid dimer and the substituted benzene rings (due to their perpendicular position) should reduce the strength of the dimer, which would lead to low frequency displacement. However, the C-I dipole of the ITMBA should have a much stronger polarizing effect on the carboxyl dimer than that of the DITMBA,

because the latter lies in a plane perpendicular to the plane of the two benzene rings.

### 3. $^{1}$ H NMR and $^{13}$ C NMR analysis of DITMBA

The $^{1}$ H NMR spectrum of DITMBA has the expected two clearly distinguishable singlet resonances at 3.91 and 3.93 ppm with an integrated intensity ratio of 6: 3, corresponding to both types of MeO groups. The broad $^{1}$ H NMR signal, centered at 8.95 ppm, was attributed to the carboxyl proton. As expected, due to the presence of a local plane of symmetry in the phenyl residue of the product molecule, only four of the six individual signals at 83.84, 140.27, 146.20 and 154.62 ppm in $^{13}$C NMR frequency were identified in the aromatic spectrum region. By analogy, the signals observed at 61.09 and 61.25 ppm were attributed to the pair of symmetric methoxy carbons and *para*-positioned MeO carbon atom.

### 4. Analysis of X-ray DITMBA contrast properties.

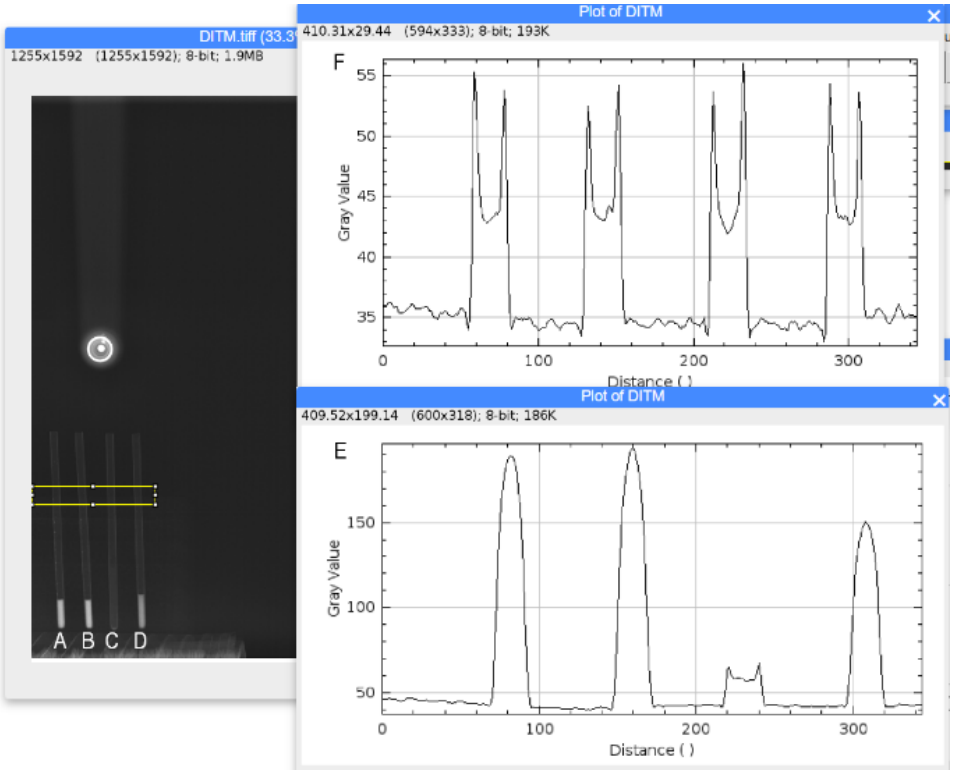



Figure 4. Radiography of A. *Ultravist*, B. *Urografin*, C. Water and D. DITMBA.

As shown in Fig. 4 the DITMBA sample exhibits an expected X-ray absorbency of approximately 90 GV. Differences in the GV index of DITMBA with those of *Ultravist* and *Urografin*, we associate with the smaller population of iodine atoms per unit working volume of a new sample. Radiographs show that DITMBA exhibited comparable but not equivalent contrast potential to that of the other two contrast agents.

In conclusion, we can say that the DITMBA sample exhibits X-ray contrast properties and can be used as a "linker" for X-ray imaging of covalently bound drugs.

## 5. Synthesis and spectral analysis of 2-iodoresorcinol, 2,4,6-triiodoresorcinol and 2,4,6-triiodophenol.

Iodination of resorcinol to 2-iodoresorcinol and 2,4,6-triiodoresorcinol was performed by a method developed by Thomsen and Torssell [4]. The synthetic methods presented by the authors have been reproduced in detail. The obtained target products were stored in the dark and at low temperature. The identity of the compounds was determined by recorded FTIR spectra (Fig. 5), where C-I bands are observed at 591 and 637 $cm^{-1}$.

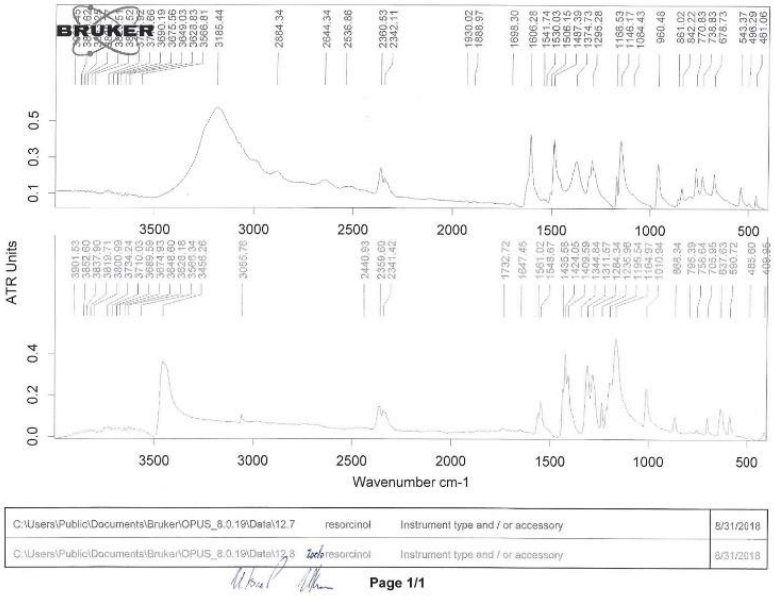


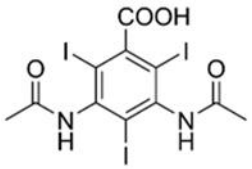

Figure 5. FTIR spectrum of starting resorcinol and riodoxol product

The identity of the newly synthesized compounds was determined by their physical indicator - melting temperature and recorded FTIR spectra, in which the presence of characteristic for C-I bonds valence oscillations is reported at 591 and 637 $cm^{-1}$. It is clear in the spectrum that one of the most characteristic bands for iodine-substituted salicylic acid is absent, namely that of the carboxyl functional in the range of 1600 to 1800 $cm^{-1}$. The similarity of the product as 2,4,6-triiodophenol was achieved on the basis of the reported total similarity (position of bands and their intensities) between the captured FTIR spectrum and that of the triiodophenol standard available in the Japanese spectral library.

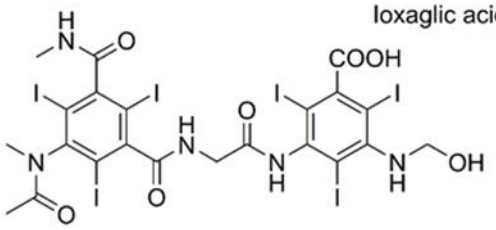
## 6. Synthesis of new triiodosubstituted aromatic acids - analogs of X-ray contrast *Amidotrizoic acid.*[5]

The present study presents an effective '*one pot'* protocol for the synthesis of triiodosubstituted aromatic (benzoic) acids - radiopaque analogues of *Amidotrizoic acid*. We used iodizing reagent ($I_2$ / $AgNO_3$), which can also be presented as safe and environmentally friendly. Moreover, suitably substituted benzoic acid methoxyderivatives were selected as appropriate targets for iodination. The total iodine content in the final products was determined by

*Volhard's* titrimetric method. Further details on the chemical structure of the reaction products were obtained by ATR FTIR and $^{1}H$ NMR spectroscopy.




Amidotrizoic acid




Ioxaglic acid

For the purposes of the study, the synthetic method described in [6] was applied in general.

In our previous studies, we obtained two iodine-containing aromatic acids derived from *Eudesmic acid* - 2-iodo-3,4,5-trimethoxybenzoic (ITMBA) and DITMBA. In both cases, however, we used the most aggressive iodizing reagent - the $I_2$/ $AgOOCCF_3$ pair. Of course, both acids were obtained in quantitative yield and with particularly high purity (> 99%). However, the method used also involves the use of the extremely hazardous and corrosive trifluoroacetic acid ($CF_3COOH$). Therefore, in the present work, we focused on developing a safer method for iodizing the title aromatic acids. In this regard, the $I_2/AgNO_3$ pair was selected here as a particularly suitable iodizing reagent. So far, only the synthesis of monoiodosubstituted compounds has been carried out with this reagent. However, in the present report, it was used for the first time in an attempt to synthesize triiodosubstituted compounds.

The performed MRI analysis did not allow us to determine the exact composition of the products thus obtained, due to the presence of several positional isomers of partially iodized acids. Contrary to expectations, however, with this technique, we were able to establish only the total consumption of the used organic precursors.

To obtain more information on the composition of the reaction products, an additional ATR-FTIR spectral analysis was performed. In general, the IR analysis was interpretively focused on the changes in the range of valence C=O oscillations (from 1650 to 1750 $cm^{-1}$).

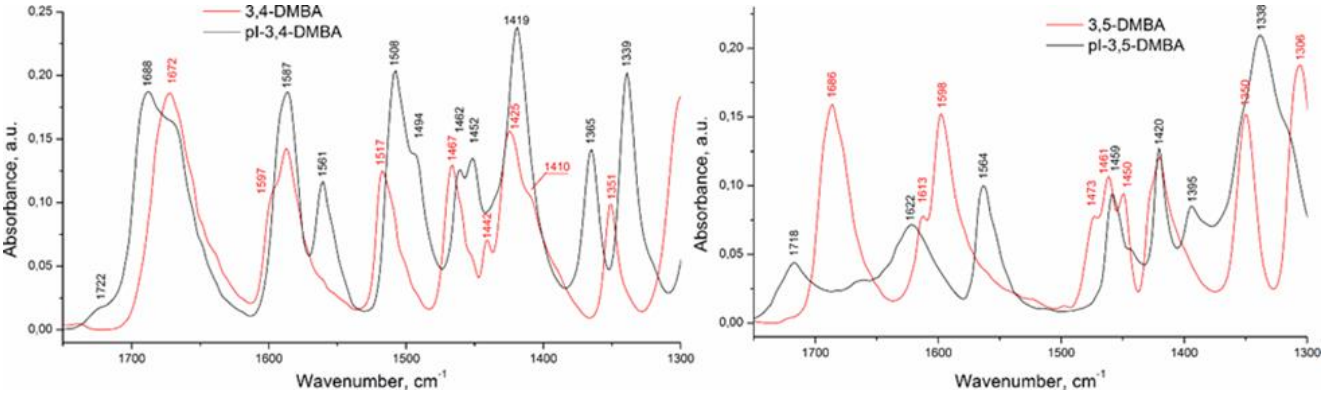



Figure 6. ATR-FTIR spectra of (A) 3,4-DMBA (red), pI-3,4-DMBA (black), (B) 3,5-DMBA (red) and pI-3,5 -DMBA (black) were taken in the area of 1750 ÷ 1300 cm $^{-1}$.

The observed shift of the frequency ν (C = O) to higher wave numbers in pI-3,4-DMBA (from 1672 to 1688 cm$^{-1}$) is associated with the presence of ortho-positioned iodine atom. Similar spectral behavior was observed in the case of the ITMBA. At this stage, however, we cannot confirm the presence of a second iodine atom in the vacant *meta* position of the aromatic 3,4-DMBA nucleus. In the same spectrum (Fig. 3A), the presence of a low-intensity band with a maximum at 1722 cm$^{-1}$ was observed. The occurrence of this absorption is associated with the presence of ortho-diode-substituted carboxyl functionality. The presence of an absorption band with a maximum at about 1672 cm$^{-1}$ in the composition of the main absorption band should be attributed to the ortho-unaffected carboxyl group. Of course, this does not mean that the most preferred iodination position, meta, is not affected. The changes that occurred throughout the IR spectrum were used as additional evidence for the total consumption of the starting aromatic acid.

Significant spectral changes in the frequency range of C=O oscillations of iodinated 3,5-DMBA were also observed (Fig. 6B). A clearly distinguishable strip with a maximum of 1718 cm$^{-1}$ was also registered. Undoubtedly, the appearance of the band in question is associated with the presence of a 2,6-diode-substituted product.

To determine the presence of exhaustive iodination of the acid in question, we performed an additional titrimetric analysis. A classical pharmacopoeial method was chosen to estimate the total iodine content.

The measured mean iodine content of pI-3,4-DMBA and pI-3,5-DMBA was found to be 2.1 ± 0.12 and 2.8 ± 0.09 mol equivalents, respectively.

The information presented here complements the content of the above spectral findings. It seems logical that the expected ortho-diode spatial interaction is likely to prevent the successful introduction of a third iodine atom into the aromatic 3,4-DMBA nucleus. Therefore, in this case the synthesis of a diode-substituted product is predominantly realized. For a pI-3,5-DMBA product, it can be said that the acid in question is obtained in almost quantitative yield.

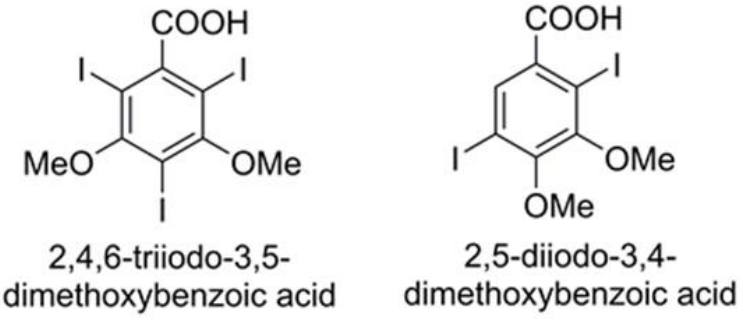



Figure 7. Main reaction products.

We continued the synthesis of polyiodosubstituted aromatic acids with the use of monomethoxy substituted benzoic acids. Suitable for this purpose were 3-methoxybenzoic (3-MBA) and 4-methoxybenzoic (4-MBA) acid. Information on the composition and iodine content of the isolated crude reaction products was again obtained by ATR-FTIR spectral and titrimetric analysis.

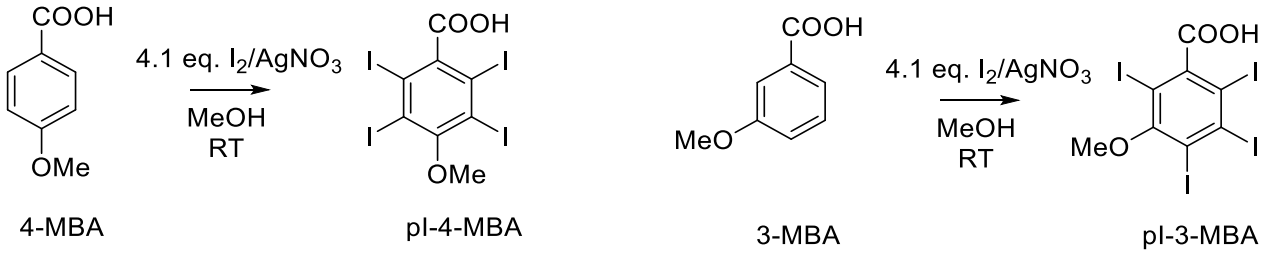



Figure 8. Synthesis of pl-3-MBA and pl-4-MBA.

The infrared spectra of the two reaction products, pI-3-MBA and pI-4-MBA, are shown in Fig.9.

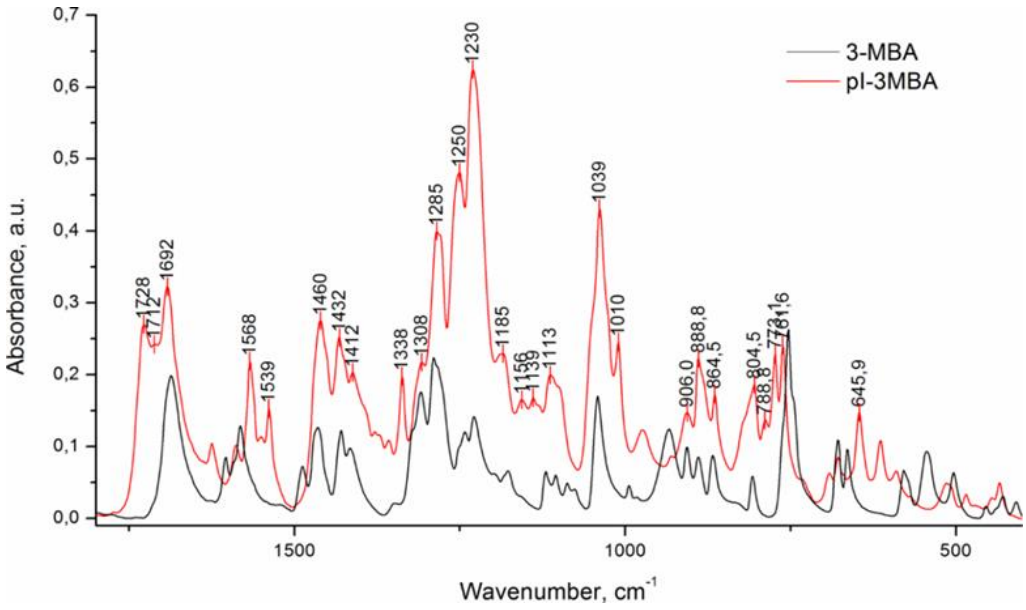



Figure 9. ATR-FTIR spectra of 3-MBA (presented alone in Annex 3) and of pI-3-MBA.

In the spectrum of pI-3-MBA, the appearance of three absorption bands in the region of valence C=O vibrations is recorded. The presence of a band with a maximum at 1728 $cm^{-1}$ is associated with the presence of an ortho-diodosubstituted product, and that at 1712 $cm^{-1}$ - with the presence of an ortho-monoiodosubstituted product. The band with a maximum at 1692 $cm^{-1}$ is considered as characteristic of the product (s) with iodine substitution in a position (*meta* or *para*) further away from the COOH group.

Indeed, the presence of at least 5 different substituted products was detected in the 1 H NMR spectrum of this product.

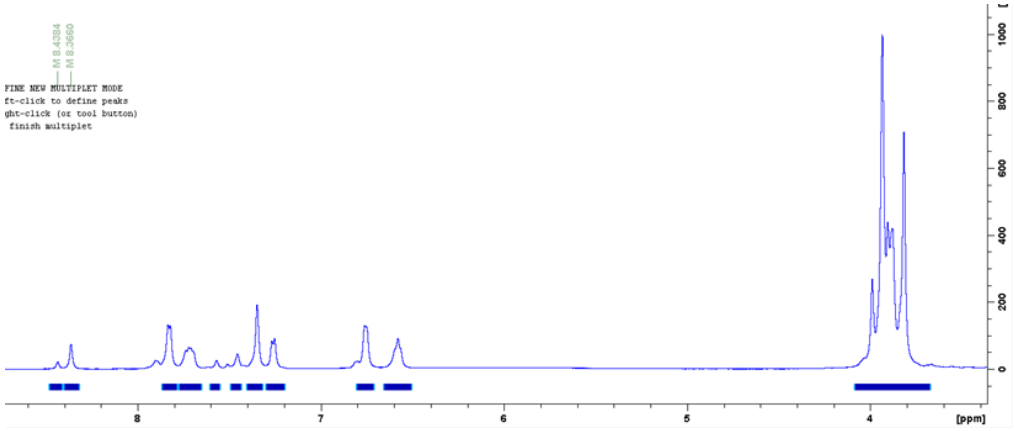



Figure 10. 1H NMR spectrum of pI-3-MBA product.

The presence of two singlet signals in a weak field, at 8.44 and 8.37 ppm, is associated with available aromatic protons placed in a strong electron-accepting environment.

In the ATR-FTIR spectrum of pI-4-MBA, the band with a maximum at 1669 $cm^{-1}$ is referred to as characteristic of *meta*-mono or diode-substituted products. The presence of a low-intensity band with a maximum at 1708 $cm^{-1}$ is associated with the insignificant presence of an *ortho*-monoiodosubstituted product.

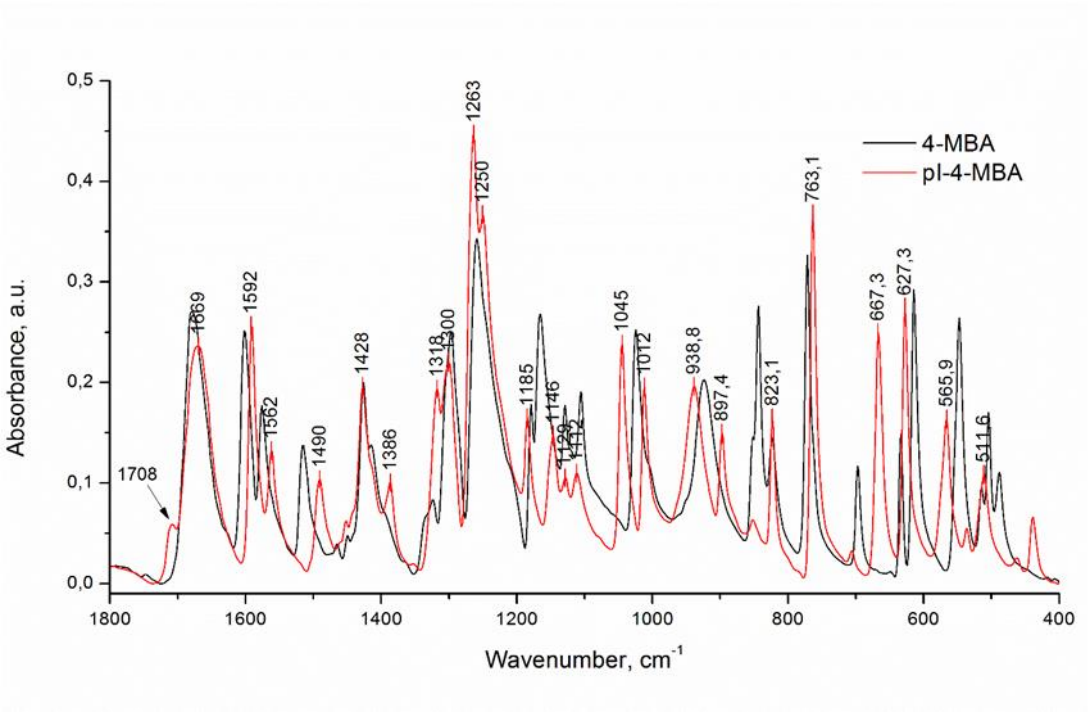



Figure 11. ATR-FTIR spectra of 4-MBA and pI-4-MBA products.

Regarding the total iodine content of these two products, titrimetric values of 3.4 iodine equivalents for the pI-3-MBA product and 2.3 equivalents for the pI-4-MBA product were determined. To prove the truth of the established titrimetric values for the total iodine content in the new products thus obtained, we conducted an additional quantitative analysis using the so-called QCM method.

The present invention relates to a method for the preparation of polyiodinated aromatics. In particular, it relates to a process including the direct iodination of 3,4- and 3,5-dimethoxysubstituted aromatic acids to its corresponding iodosubstituted derivatives.

In general, however, this methodology proved to be suitable only for the synthesis of 2,4,6-triiodo-3,5-dimethoxybenzoic acid.

## 7. Analysis of the results for total iodine content obtained by the method of QCM (Quartz Crystal Microbalance).

In the present study, we used the QCM method as an alternative (physical) method for determining the total iodine content of the organoiodic compounds we obtained.

The method, in essence, is similar to other thermogravimetric methods, which also take into account changes in the mass of the sample as a function of the imposed temperature changes. The piezoelectric method used here can analyze, in addition, of course, after applying an appropriate analysis, not only the above-mentioned functional dependence $\Delta m=f(t)$ but also the phase changes caused by the ongoing thermodestruction processes (such as which should be observed in any aromatic deiodination reaction).

All analyzes were performed in dynamic mode. This allowed us to track the change in the frequency of the quartz-crystal microbalance in a series of experimental replicates, to analyze, in addition, the thermogravimetric profile and to report sharp dynamic changes in the viscosity of the studied samples (or their so-called viscoelastic characteristics).

The successful course in this type of measurements was achieved after the formation of a homogeneous layer of each analyte on the working Au electrode surface. To obtain a stable output signal ($f_1$), the analytes were applied in the form of toluene solutions, which were evaporated to dryness (in an Ar stream).

After establishing a constant frequency signal, the samples were subjected to thermal activation at 350°C by means of a warm air jet generated by a programmable heater. The stages of sample preparation were performed in a specially designed pyrex cuvette, in the internal free volume of which the quartz plate loaded with an organic sample was placed.

The effect of heating the quartz plate is manifested in the sharp rise and subsequent loss of the resonant signal. The registered effect is most likely due to additive changes resulting from phase transitions (from solid to liquid) and thermo-associated losses in the masses of the samples associated with the destruction of their unstable C-I bonds and the release of elemental iodine.

Within this stage, but after a period of high-temperature exposure, the QCM "restores" its resonant frequency; falling back into its operating (10 MHz) mode.

It is logical to assume that the "frequency losses" that have occurred should be perceived as weight/mass. Experimental confirmation of this can be found in the established "new" natural frequency of the heat-derived QCM plate. We associate the reported frequency changes in this interval only with the changes in the masses of the studied substances.

Each analyte was subjected to additional heat treatment under identical conditions (temperature 350°C; stream of pre-dried argon fed at 100 mL/min). This activation process was repeated three times. Activation repetitions aim to minimize the expected errors associated with the partial deiodination of the samples.

Table 2 shows the changes in the operating frequency of the resonator QCM after loading with the tested samples and their high-temperature activation ($f_2$).

Regarding the results obtained for the other four iodinating acids, pI-3-MBA, pI-4-MBA, pI-3,4-MBA and pI-3,5-MBA, it can be said that a very good match is again reported. with the established titrimetric for them, namely:

3.4 iodine equivalents for pI-3-MBA (QCM), compared to 3.4 (titrimetrically determined)

2.77 iodine equivalents for pI-4-MBA (QCM), compared to 2.3 (titrimetrically determined)

2.06 iodine equivalents for pI-3,4-MBA (QCM), compared to 2.1 (titrimetrically determined)

2.75 iodine equivalents for pI-3,5-MBA (QCM), compared to 2.8 (titrimetrically determined)

In conclusion, we can summarize that the additional method used by us for estimating the iodine content can be used with extremely high accuracy and sensitivity with respect to complex iodine products.

## 8. Synthesis of *meta*-terphenyl derivatives.

The synthesis of biphenyl and *meta*-terphenyl derivatives was performed according to an adapted synthetic procedure, which does not require the use of Pd-containing ligands. The synthesis of the desired products was carried out in an aqueous solution in the presence of polyethylene oxide (PEO) [7,8,9,10].

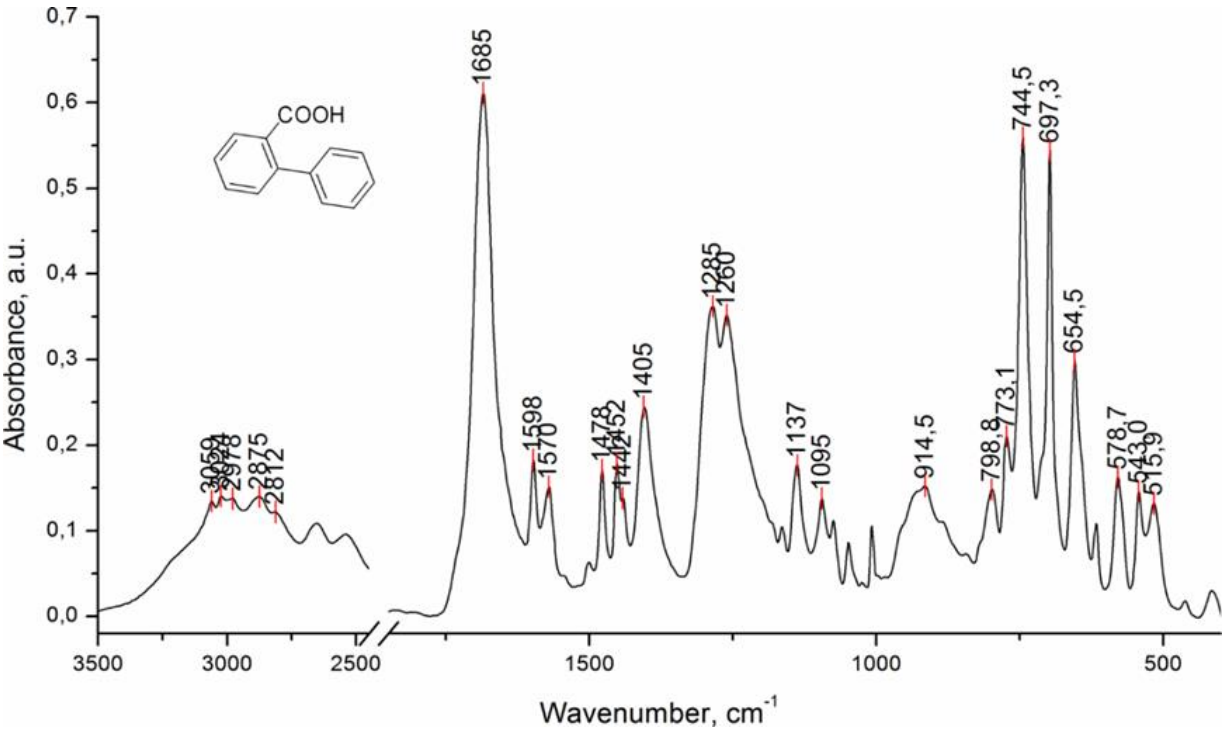



Figure 12. ATR-FTIR spectrum of [1,1'-biphenyl]-2-carboxylic acid

The identity of the acid in question was determined by ATR-FTIR analysis. The acid spectrum is shown in Figure 5.

In the Japanese Spectral Database (SDBS), for [1,1'-biphenyl] -2-carboxylic acid (listed under its synonym 2-biphenylcarboxylic acid), the following IR absorption parameters are presented (tabularly): **3066**, 3027, 3017 , **2904**, 2882, 2814, 2679, **2663**, 2653, **2575**, 2646, 1696, **1685**, **1598**, **1568**, **1480**, **1454**, **1442**, **1412**, **1402**, 1306, **1296**, 1284, **1268**, 1146, **1139**, **1097**, **1009** , **919**, **800**, **777**, 762, 743, **701**, 666 $cm^{-1}$. The tabular values marked in bold coincide with those defined in the spectrum taken by us. In some places, minimal deviations are reported due to the differences in the two spectral operating modes - transmission and ATR.

Undoubtedly, the adjacent COOH group does not interfere, sterically, with the progress of CC coupling. It is logical to expect that such an effect should not be observed in cases where DITMBA is used as a reactant.

The research with 2-iodobenzoic acid was continued with the use of the second organoborne reactant - 4-methoxyphenylboronic acid. The identity of the acid in question was determined by ATR-FTIR analysis. The acid spectrum is shown in Figure 13:

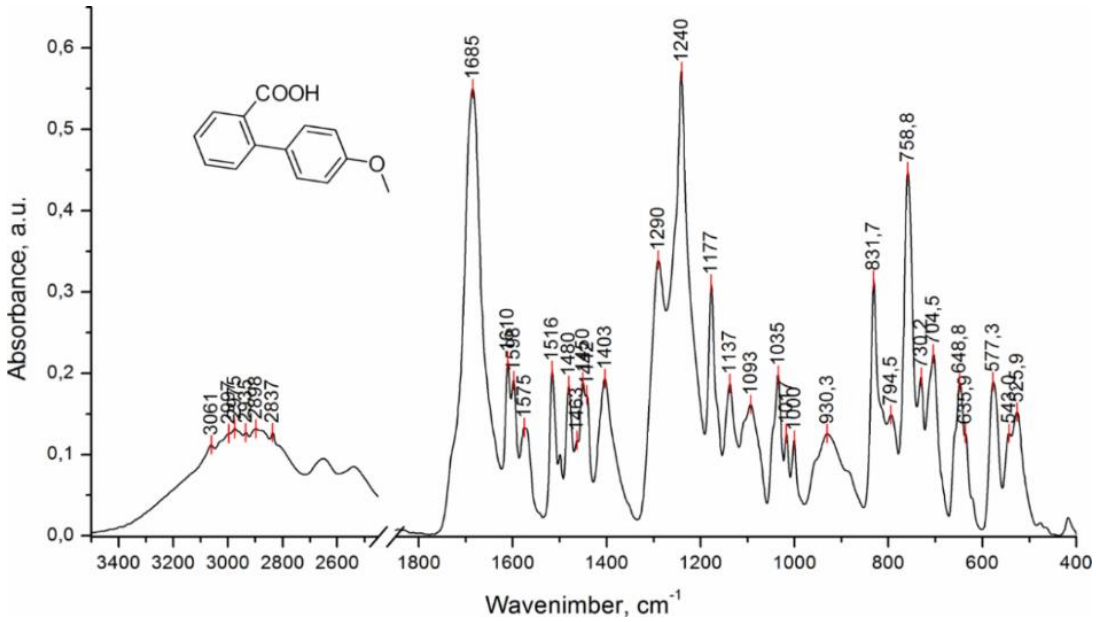


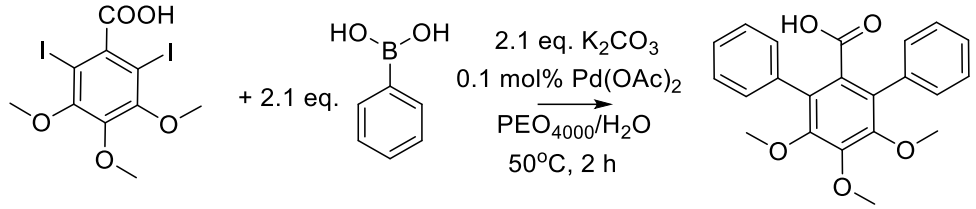

Figure 13. 4'-methoxy-[1,1'-biphenyl]-2-carboxylic acid ATR-FTIR spectrum.

FTIR analysis of the obtained *meta*-terphenyls was performed on the basis of a comparative analysis with that already described for DITMBA. The presence of the characteristic MeO group oscillations at 1017 and 1035 $cm^{-1}$ is recorded in the recorded spectrum. *In-plane* deformation oscillations of the methyl group are associated with the present medium-intensity band at 1177 $cm^{-1}$, and valence symmetric and asymmetric CH oscillations - with those at 2837 and 2898 $cm^{-1}$. Differences in the degree of substitution of the second phenyl residue are the reason for the appearance of a larger number of absorption bands in the range from 1400 to 1650 $cm^{-1}$. In the same zone, we should expect the scissor deformation oscillations of the CN bonds that are part of the methyl residue.

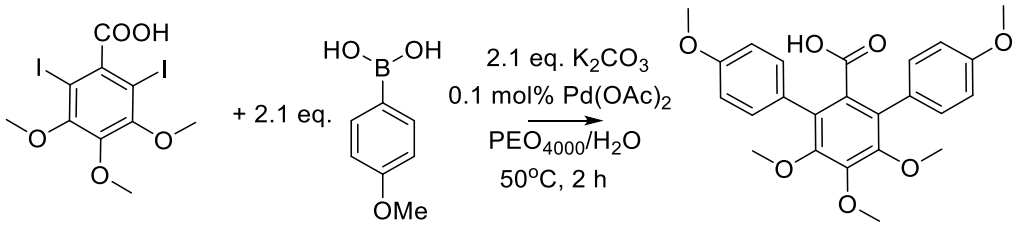
The vibrational behavior of the COOH functional is similar to that of the COOH residue of 4'-methoxy- [1,1'-biphenyl] -2-carboxylic acid.

For the synthesis of meta-terphenyl derivatives, we applied similar reaction conditions (Schemes 1 and 2). The obtained products were isolated by column chromatography. As expected impurities in the composition of each reaction product, we found the homocoupled product of the arylboronic acid used, namely biphenyl and 4,4'-dimethoxybiphenyl (4,4'-bianisol). Therefore, the steric effect

cannot be completely ignored in the case of ortho-iodine-substituted benzoic acids.




Scheme 1. Synthesis of 4',5',6'-trimethoxy-[1,1':3',1''-terphenyl]-2'-carboxylic acid.




Scheme 2. Synthesis of 4,4 ', 4' ', 5', 6'-pentamethoxy-[1,1':3',1''-terphenyl]-2'-carboxylic acid.

The identity of the *meta*-terphenyls was established by ATR-FTIR and $^{1}H$ and $^{13}C$ NMR analysis. The composition of the obtained by-products was analyzed by ATR-FTIR spectral analysis.

ATR-FTIR spectra of the two main reaction products, 4',5',6'-trimethoxy-[1,1':3',1''- terphenyl]-2'-carboxylic acid and 4,4',4''5',6'-pentamethoxy-[1,1':3',1''-terphenyl] -2'-carboxylic acid are shown in Figures 14 and 15.

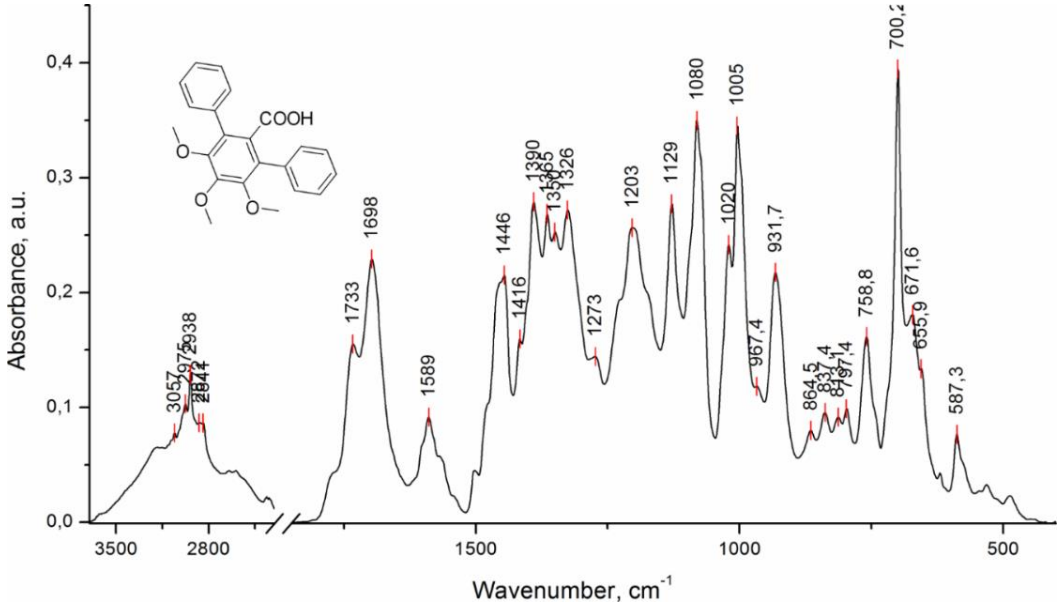



Figure 14. ATR-FTIR spectrum of 4',5',6'-trimethoxy-[1,1':3',1"-terphenyl]-2'-carboxylic acid.

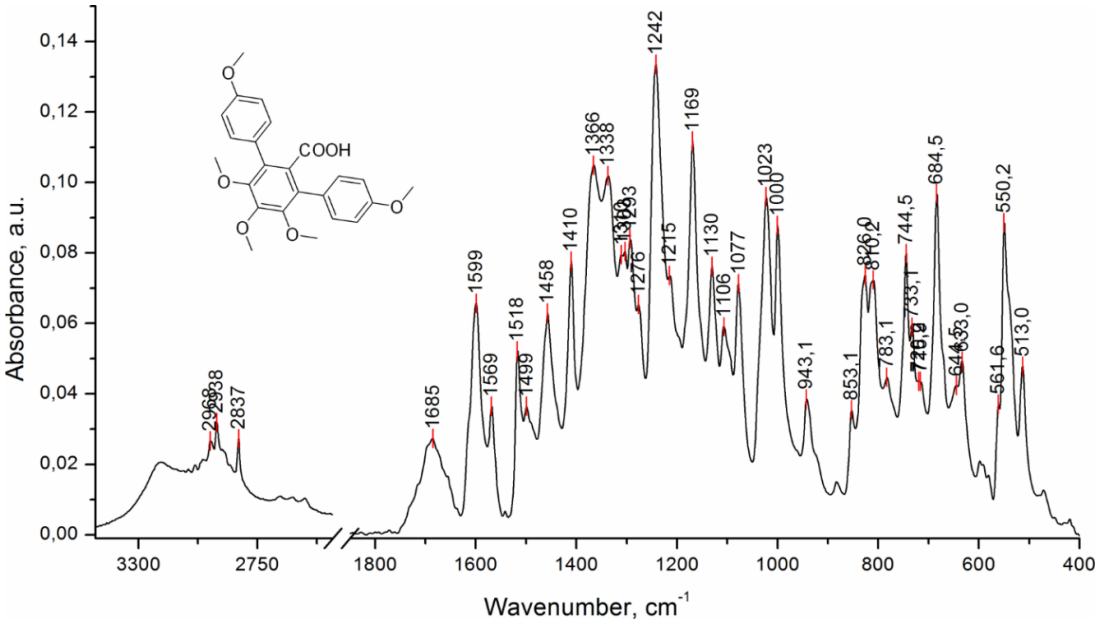



Figure 15. 4',4",5',6'-pentamethoxy-[1,1':3',1"-terphenyl]-2'-carboxylic acid ATR-FTIR spectrum

In the ATR-FTIR spectra of 4',5',6'-trimethoxy-[1,1':3',1"-terphenyl]-2'-carboxylic acid, the bands characteristic of valence asymmetric C-H oscillations are observed at 2975 and 2938 $cm^{-1}$, and for symmetric valence vibrations - at 2844 $cm^{-1}$. In the spectrum of the pentamethoxy-containing analogue, 4,4',4",5', 6'-pentamethoxy-[1,1':3',1"-terphenyl]-2'-carboxylic acid, equivalent to those indicated bands are observed at 2968, 2938 and 2837 $cm^{-1}$. The bands characteristic of asymmetric and symmetric CH strain oscillations of 4',5',6'-trimethoxy-[1,1':3',1"-terphenyl]-2'-carboxylic acid are found to overlap in the composition of composite strip with a maximum at 1446 $cm^{-1}$ and at 4,4',4",5',6'-pentamethoxy-[1,1':3',1"-terphenyl]-2'-carboxylic acid - at 1458 $cm^{-1}$. Observed high intensity bands at 1005 and 1020 $cm^{-1}$ (in the ATR-FTIR spectrum of 4',5', 6'-trimethoxy-[1,1':3',1"-terphenyl]-2'-carboxylic acid) and the low-intensity band entering the arm of a complex band with a maximum at 1273 $cm^{-1}$ are attributed to symmetric and asymmetric valence O–$CH_3$ oscillations as well as to $C_{Ar}$-$OCH_3$ oscillations, in the pentamethoxy-containing analogue equivalent to those here very intense stripes, we observe at 1000 and 1023 $cm^{-1}$) and with low intensity at 1276 $cm^{-1}$).

The bands with maxima at 797 and 758 $cm^{-1}$ are attributed to the expected additional "C-$OCH_3$ valence" and C-$OCH_3$ deformation oscillations. For 4,4',4", 5',6'-pentamethoxy-[1,1':3',1"-terphenyl]-2'-carboxylic acid, the corresponding bands are reported at 744 and 733 $cm^{-1}$ (for valence C-$OCH_3$ and deformation C-$OCH_3$ oscillations), and for rocking deformation oscillations - at 1169 and 1130 $cm^{-1}$.

As a rule, the shift of the valence C = O oscillations in the direction of larger wave numbers is associated with the absence of conjugation between the carboxyl functional and its adjacent aromatic base; as is the case with DITMBA.

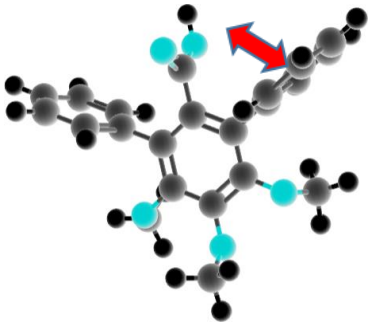


Figure 16. Illustration showing the expected optimal molecular geometry of 4', 5',6'-trimethoxy-[1,1':3',1"-terphenyl]-2'-carboxylic acid.

The possibility of the association of these acids in carboxyl dimers predetermines the appearance of a symmetrical center. The latter, in turn, gives rise to two types of COOH oscillations - in-phase and out-of-phase. In the IR spectrum of 4',5',6'-trimethoxy-[1,1':3',1"-terphenyl]-2'-carboxylic acid, out-of-phase (asymmetric) vibrations are responsible for the occurrence on the absorption band with a maximum at 1326 $cm^{-1}$, a in-phase and out-of-phase C-O-H vibrations - at 1416 and 932 cm-$^{1}$; in the spectrum of 4,4',4",5',6'-pentamethoxy-[1,1':3',1" - terphenyl] -2'-carboxylic acid these vibrations were recorded at 1338, 1410 and 943 $cm^{-1}$.

In the presented $^{1}H$ NMR spectrum (Fig. 10) of 4',5',6'-trimethoxy-[1,1':3',1" - terphenyl]-2'-carboxylic acid, the expected two highly intense signals are observed for $CH_3O$ protons at 3.99 and 3.63 ppm.

The integral intensities of the two signals correspond to the expected ones, namely three protons for the methyl group in the 4′ MeO group and six protons for the methyl groups in the two MeO groups located at the 3′ and 5′ positions. The signals of MeO protons inherent in the expected biphenyl impurity (indicated by red arrows in the spectrum under consideration) are recorded in the same frequency range.

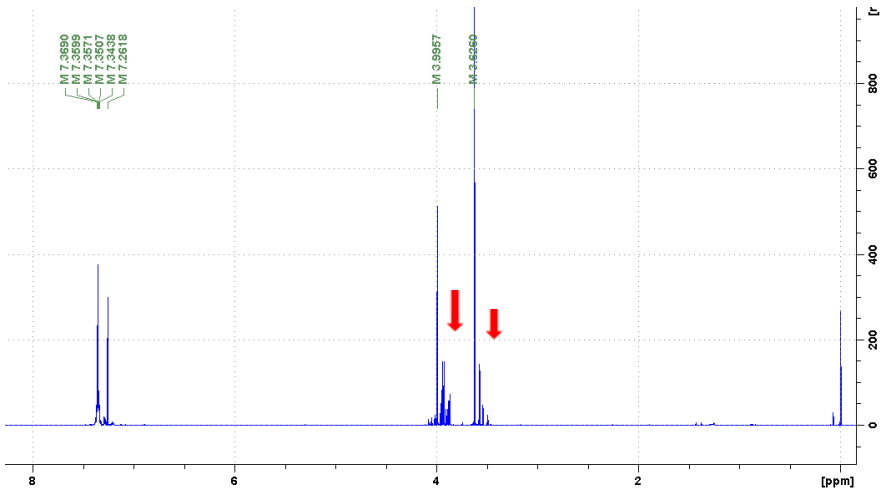



Figure 17. $^{1}$H NMR spectrum of 4',5',6'-trimethoxy-[1,1':3',1"-terphenyl]-2'-carboxylic acid.

Additional evidence for the successful synthesis of the desired 4',5',6'-trimethoxy-[1,1': 3',1"-terphenyl]-2'-carboxylic acid was obtained by $^{13}$ C NMR analysis.

The expected presence of 11 carbon signals is recorded in the spectrum (Fig. 10). To illustrate the presence of some more closely localized signals, in a separate figure, an element of the same spectrum was presented, but in the range of 120 to 140 ppm

However, the presence of a low-intensity signal at 170.8 ppm, characteristic of the present COOH carbon atom, was recorded in the spectrum. In a stronger field, the signals inherent in the other carbon atoms of the centrally positioned terphenyl ring can be detected, namely: δ 150.8 (C1'; C3', s), 147.8 (C5 ', s), 128.1 (C2) , s) and 127.6 (2C4 ') ppm. The signals for the four symmetric atoms in the composition of the terminal phenyl residues, 2C2, 2C2 ", are found with reciprocal intensity at 127.99 ppm, and those for 2C3, 2C3" atoms - at 129.7 ppm. The presence of the other two pairs of atoms - C1, C1 " and C4, C4" - is determined by the present signals at 135.4 and 129.9 ppm. The signals of MeO groups are found in a strong field, at 61.16 and 61.14 ppm.

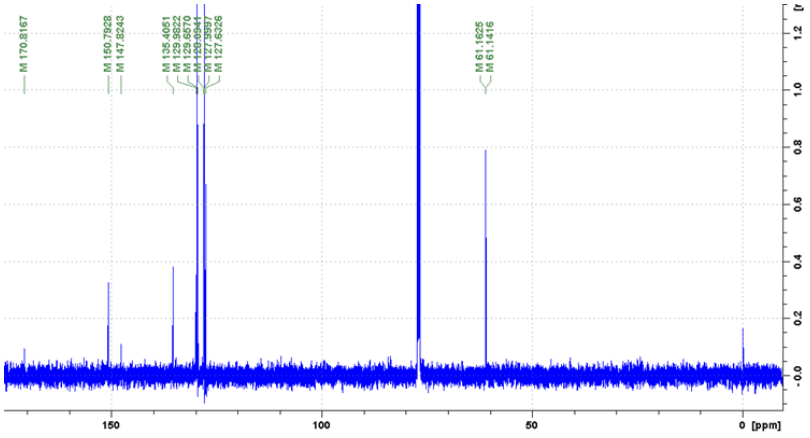



Figure 18. $^{13}$C NMR spectrum of 4',5',6'-trimethoxy-[1,1':3',1''-terphenyl]-2'-carboxylic acid

In the presented $^{1}$H NMR spectrum, the expected singlet signals for $CH_3$ protons from the composition of MeO groups at 3.62, 3.84 and 3.99 ppm were observed. The integral intensity corresponds to expectations, namely - three protons for the methyl group of the MeO-C4' residue and six protons for the methyl groups of the MeO groups located at positions 3' and 5 'and respectively - six protons for $CH_3$ residues of the composition of the two telehel MeO groups located at positions 4 and 4''. The presence of three signals is detected in a weak field. The signal at 7.26 ppm is characteristic of the solvent used - $CDCl_3$, and those at 6.91, 6.93 and 7.29, 7.31 - are attributed to the hydrogen protons of the two terminal phenyl residues.

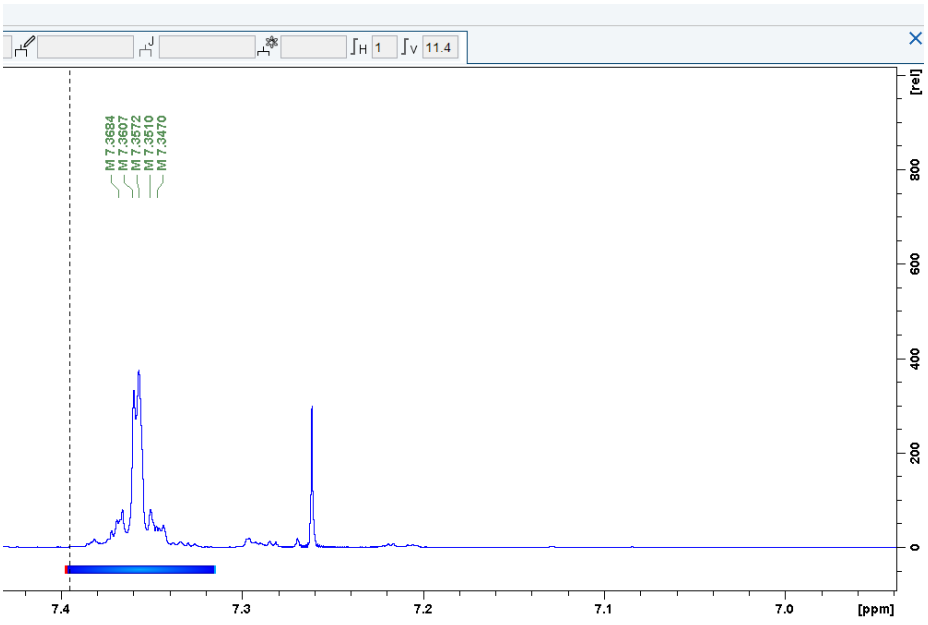



Figure 19. $^{1}$H NMR spectrum of 4',5',6'-trimethoxy-[1,1':3',1"-terphenyl]-2'-carboxylic acid in the range of 6.5 to 7.5 ppm

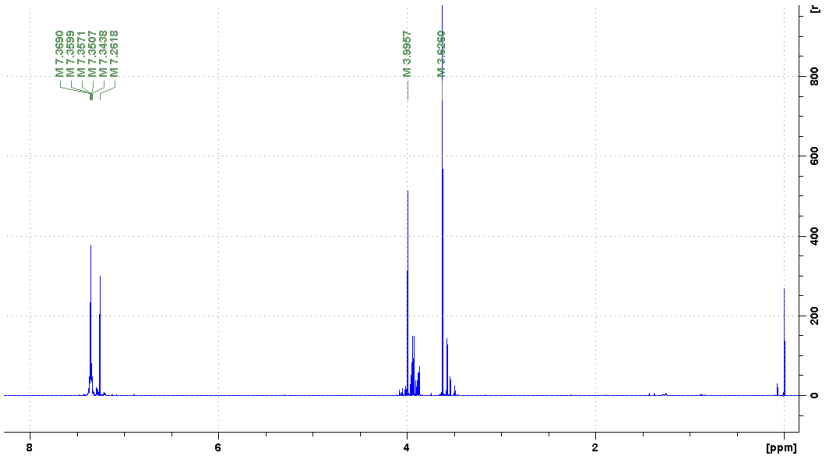



Figure 20. $^{1}$H NMR spectrum of 4',5',6'-trimethoxy-[1,1':3',1"-terphenyl]-2'-carboxylic acid

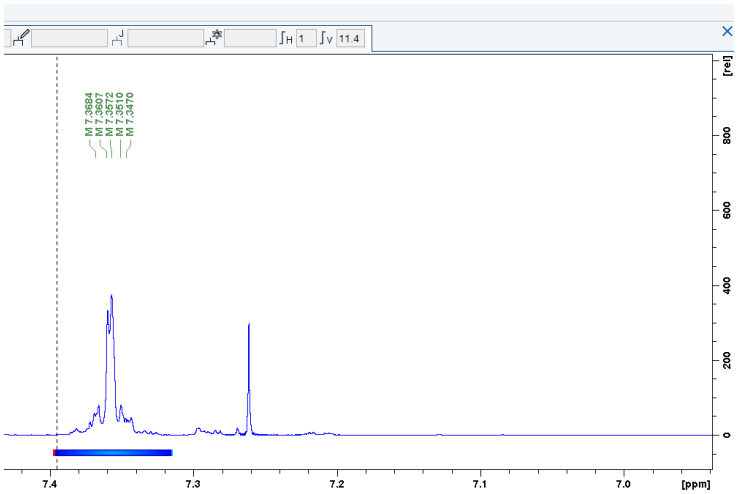
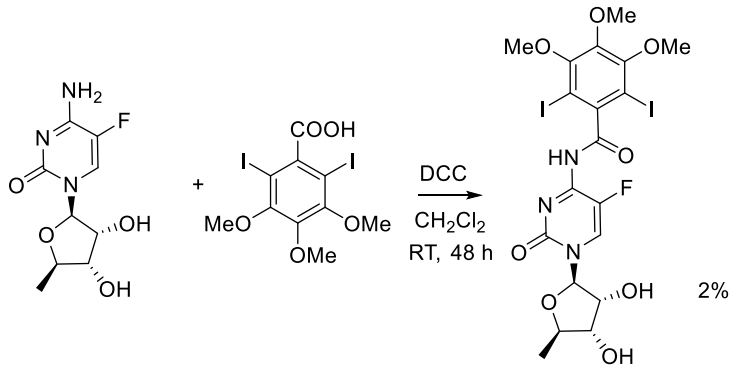



Figure 21. $^{1}$H NMR spectrum of 4',5',6'-trimethoxy-[1,1':3',1''-terphenyl]-2'-carboxylic acid in the range of 6.5 to 7.5 ppm

## 9. Synthesis of diode-functionalized *5'-fluorocytidine* analog.

The synthesis of N-(1-(3,4-dihydroxy-5-methyltetrahydrofuran-2-yl) -5-fluoro-2-oxo-1,2-dihydropyrimidin-4-yl)-2,6-diiodo-3, 4,5-trimethoxybenzamide was reacted with DITMBA and 5'-deoxy-5-fluorocytidine:




## 10. DITMBA cocrystals with *Nitrofural, Metronidazole*.

The potential of DITMBA was also investigated in the synthesis of cocrystals with four drugs - *Nitrofural, Nifuroxazide, Nitrofurantoin* and *Metronidazole.*

Unfortunately, in the process of co-crystallization of DITMBA with Nitrofurantoin, Nifuroxazide, the drugs precipitated in the form of crystalline precipitates in a period of less than 1 hour. The presence of single Nitrofural crystals (<5%) was reported in the synthetic Nitrofural solution. The latter were

carefully separated from the reaction solution by filtration. After approximately 24 hours, the presence of needle crystals was detected in the volume of the co-crystallization Nitrofural-DITMBA solution. The crystals were isolated from the solution using a pipette. We dried the latter on the surface of filter paper. The crystals were transferred to a glass petri dish and subjected to further drying in a desiccator over P2O5. The ATR-FTIR spectrum of the crystals thus obtained is shown in Figures 22 and 23.

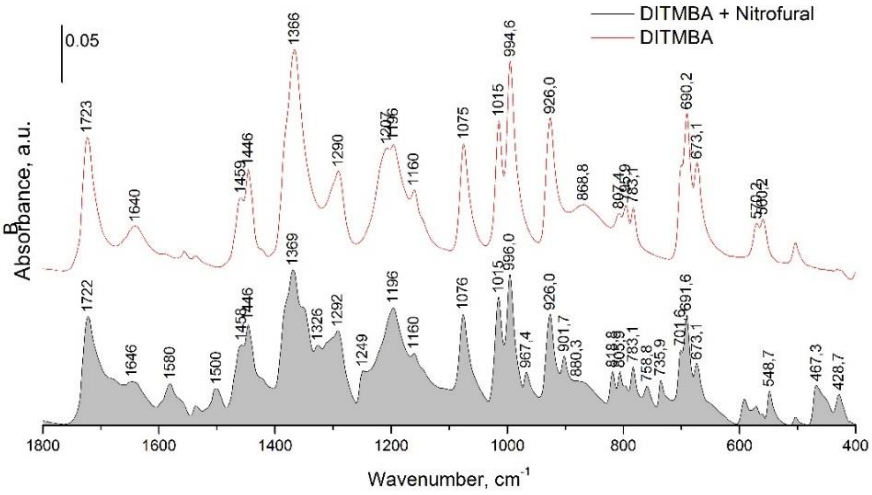



Figure 22. ATR-FTIR spectra of DITMBA and the resulting DITMBA-*Nitrofural* crystal mass.

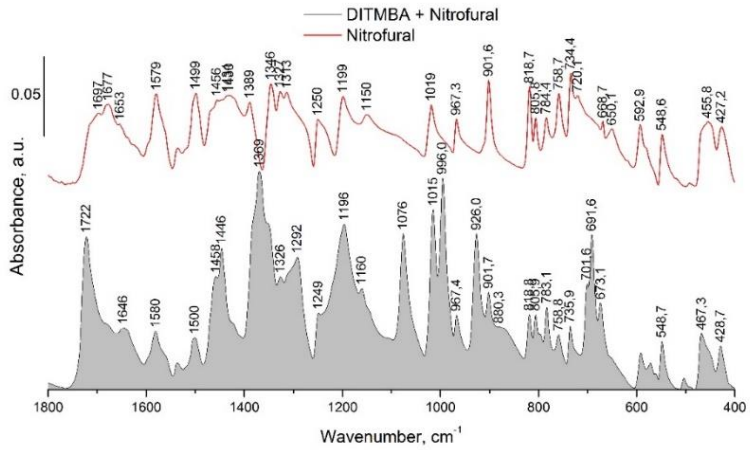



Figure 23. ATR-FTIR spectra of *Nitrofural* and the resulting DITMBA + *Nitrofural* crystal mass.

From the presented, it can be seen (Figs. 15 and 16) that in the spectrum of the separated DITMBA-*Nitrofural* crystals bands characteristic of both components are observed.

The presented powder X-ray diffraction pattern shows that the studied sample is a polycrystal organized mainly by crystals of DITMBA acid and *Nitrofural*.

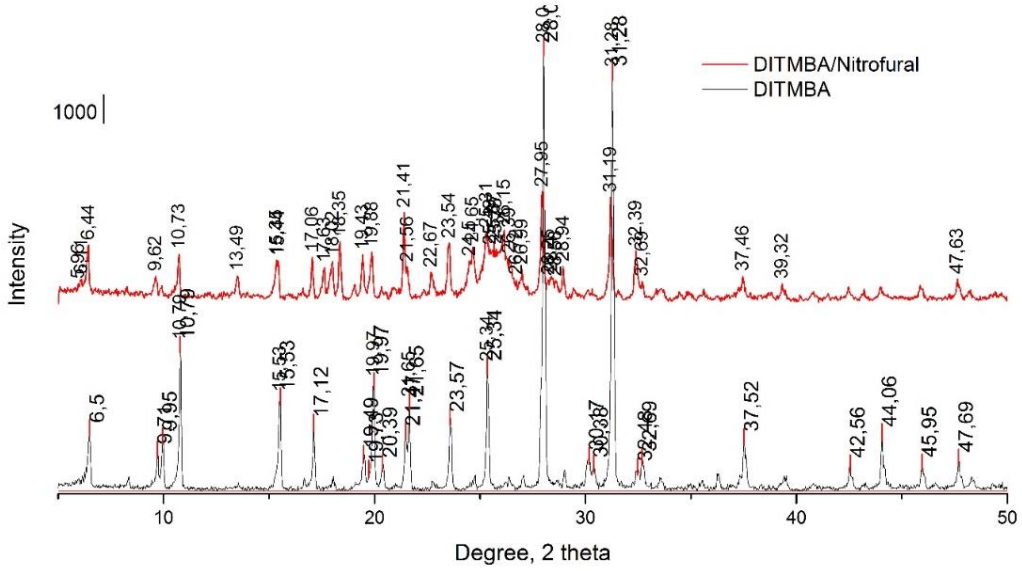



Figure 24. Radiograph of the DITMBA sample and the resulting DITMBA + *Nitrofural* crystal mass.

## 11. *Allium cepa* test to assess the potential genotoxicity and cytotoxicity of DITMBA

In 1938, A. Levan developed a bioassay to study the effect of colchicine on germinating onion roots. As a test object, the author uses a meristem of the plant species Allium cepa L., due to the fact that this species is particularly sensitive to various chemicals, predetermined by its insignificant chromosome number (2n = 16).

The analysis of actively dividing meristem cells allows to identify disorders in the process of normal cell division, such as the presence of chromosomal aberrations in mitotic cells, impaired structure and/or functionality of their dividing spindle, the presence of micronuclei in interphase cells and other effects.

Today, the Allium cepa test is a well-established and widely used method for determining the cyto- and genotoxicity of new and known substances. That is why the WHO recommends this method as a standard in cytogenetic monitoring and as an alternative to in vivo tests in experimental animals.

This test is cheap, fast, and easy to perform. It is highly sensitive and allows the reporting of positive toxic effects, even when the tested samples with other methods do not detect genotoxic results.

An example is the tragedy caused by Thalidomide, which could have been avoided if this test had been applied.

The test is reproducible, easy to perform and low cost. It also provides the ability to directly observe and study chromosomal aberrations and disorders in the cell cycle, including all forms of aneuploidy.

The test also correlates with other test systems, such as with human lymphocytes, lysogenic bacteria, hamster cell line V79, etc. *Fiskesjö* (1985) research shows the importance of the Allium cepa test in another aspect. They prove that *Allium cepa* cells contain a specific system of oxidases (the mixed-function-oxidases or the MFO-system), capable of metabolizing even polycyclic hydrocarbons. In this way, these cells are able to activate so-called promutagens and complement the ability of the test to analyze potential genotoxicity. The test has also proven to be an ideal bioindicator for primary cytotoxicity screening.

That is why the test combines two main goals: assessment of toxicity and mutagenicity. Toxicity correlates with the degree of growth inhibition and disturbances in the mitotic process (cycle), determined by the indicators mitotic index (index for each mitotic stage), percentage of mitotic cells with defects and percentage of impaired interphase cells.

That is why the test combines two main goals: assessment of toxicity and mutagenicity. Toxicity correlates with the degree of growth inhibition and disturbances in the mitotic process (cycle), determined by the indicators mitotic index (index for each mitotic stage), percentage of mitotic cells with defects and percentage of impaired interphase cells.

Mutagenicity is considered to be the most critical genotoxic mechanism, as it is directly related to damage to DNA molecules. The genotoxic potential is determined by quantification of each chromosomal (including nuclear) abnormality (type and frequency of chromosomal aberrations), as well as analysis

of the presence of micronuclei. The duration of treatment is 24 hours and/or 48 hours, including one and/or two cell revolutions (cycles), respectively.

Therefore, the cytotoxicity and genotoxicity of our newly synthesized compound (DITMBA) were determined using the "publicly available" (easily feasible and low cost) Allium test (Fig. 26). To rule out the possibility of the presence of artifacts, we selected active biological samples (bulbs of the plant Allium cepa L.) with provthe en origin and biological purity.

The duration of exposure to the test substances was studied for a period of 24 hours in which a complete life (cell) cycle takes place for the selected cellular species (cell line). Two controls were used in the assay: positive with 5-Fluorouracil and negative with distilled water. The newly synthesized substance was administered in two working concentrations - 0.3 and 0.16 mg/mL, respectively.

Figure 25. Photographs illustrating the steps of the Allium test.

As expected, analysis with distilled water (negative control) showed normal rupture of intercellular connections (cell separation) and the presence of multiple cells in the mitosis stage. Meristematic cells dividing in meta- and anaphase stages were also observed (Fig. 26):

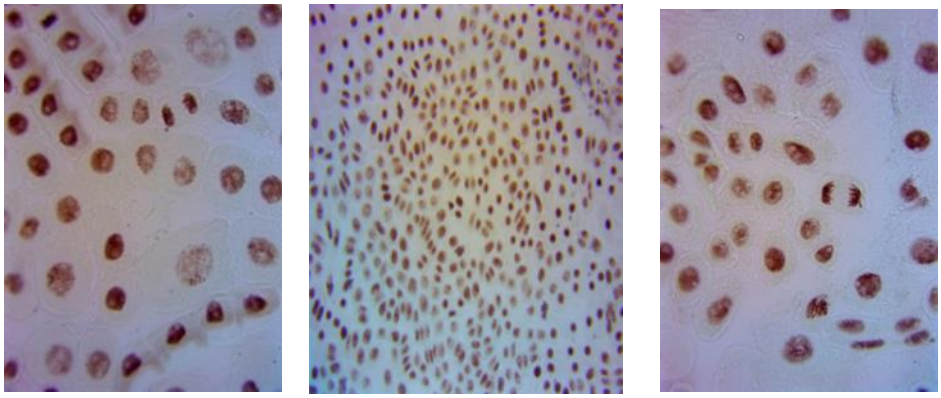


Figure 26. Normally dividing mitotic cells from the root meristem of Allium cepa L. observed in the negative control with distilled water.

However, in the positive control, the presence of dense areas of inactive populations of meristem cells with dark-colored nuclear fragments from the period of active cell division was detected (Fig. 27). Here, suppression of mitotic activity is reported as a sign of the cytotoxic effect caused by the studied component.

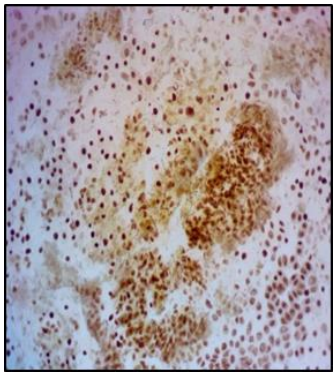


Figure 27. Mitotic cells of the root meristem of Allium cepa L. after exposure to 5-Fluorouracil.

Samples treated with a solution of the newly synthesized substance (in a higher concentration) have a "compacted" population of cells - a population that does not allow the lysis of intercellular contacts. Therefore, in this sample, it was not possible to take into account the following cytogenetic parameters - mitotic index, phase index for each mitotic phase, the percentage of abnormal mitoses and the percentage of abnormal interphase cells (Fig. 28).

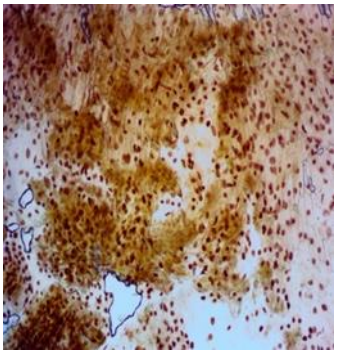


Figure 28. Mitotic cells of the root meristem of Allium cepa L. after exposure to DITMBA at a concentration of 0.3 mg / mL.

However, the treated cells with a lower concentration of the newly synthesized substance allowed us to detect the presence of normally dividing cells in the prophase and metaphase phases, and only in isolated cases - disorders related to the torn chromosome bridge indicated by the arrow. The probability of the manifestation of genotoxic action of the newly synthesized substance is also rejected on the basis of the reported absence of the so-called micronuclei (Fig. 29).

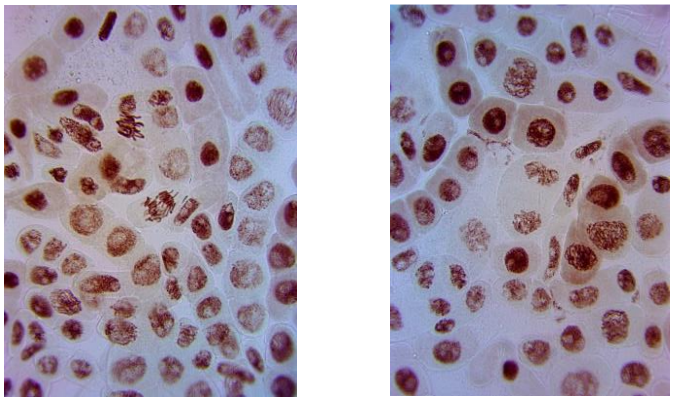


Figure 29. Mitotic cells of the root meristem of Allium cepa L. after exposure to DITMBA at a concentration of 0.16 mg / mL.

The analysis performed by us showed that at the applied concentrations the diodosubstituted molecule exhibits a dose-dependent cytotoxic effect. The absence of micronuclei in the cells treated with it ruled out the possibility of its direct action (toxic) on DNA.

## 12. Antimicrobial activity.

Microbiological tests were performed with all iodine-containing substances synthesized by us according to the established recommendations of EUCAST (The European Committee on Antimicrobial Susceptibility Testing).

The low water solubility of the organoiodic compounds synthesized by us was the reason to modify the well-known and established microbiological methods used mainly for the analysis of water-soluble antibiotics. The introduced innovation allowed us to study in detail and with experimental accuracy and reproducibility all iodine-substituted compounds compared to our selected crops. The modification included the application of a technique with which we successfully deposited the analyzed substances on a cellulose disk, namely: pipetting different amounts of solutions of known concentrations (dichloromethane solutions) on inert paper and drying with warm air in a sterile box. This approach allowed us to process the studied biological strains with specific amounts of each substance and evaluate their activity against them. All microbiological tests were repeated three times.

Figure 30. Figures illustrating the results of biological tests performed according to the Bauer-Kirby Disc-Diffusion Method (DDM).

In order to study and compare the behavior of the selected microorganisms in relation to the organoiodic substances synthesized by us, we determined the

characteristic clinical indicator - MIC. MICs are a standard guide for the susceptibility of a microorganism to any antimicrobial reagent. The minimum inhibitory concentration (MIC) for each substance was determined by the modified method. We evaluated the truth and reliability of this method with the help of the generally accepted microbiological reference method - the method of serial dilutions. The rhodoxol molecule was selected as a control - a drug substance with proven antiviral, antifungal and antibacterial activity.

**1. Antimicrobial activity, determined by our modified method of serial dilution** (Tables 3, 4 and 5)

Table 1. Table illustrating the activity of DITMBA, iodoresorcinol (IRes), 2,4,6-triiodophenol (TIPh) and riodoxol (RiOL) against three different strains of *Candida albicans*, in which the presence of antifungal activity, and with the operator (-) the lack of activity is reflected.

| ***Candida albicans*** | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| DITMBA | Strain | | | IRes | Strain | | | TIPh | Strain | | | RiOL | Strain | | |
| | 1 | 2 | 3 | | 1 | 2 | 3 | | 1 | 2 | 3 | | 1 | 2 | 3 |
| 3.0 мг | - | - | - | 0.4 µгр | + | + | + | 0.6 мг | - | - | - | 0.4 мг | + | + | + |
| 4.0 мг | - | - | - | 0.5 µгр | + | + | + | 1.2 мг | + | + | + | 0.45 мг | + | + | + |
| 6.0 мг | - | - | - | 0.6 µгр | + | + | + | 1.8 мг | + | + | + | 0.5 мг | + | + | + |
| | | | | 0.7 µгр | + | + | + | 2.4 мг | + | + | + | 0.55 мг | + | + | + |
| | | | | 0.8 µгр | + | + | + | | | | | 0.6 мг | + | + | + |

Table 2. Table illustrating the activity of DITMBA, iodoresorcinol (IRes), 2,4,6-triiodophenol (TIPh) and riodoxol (RiOL) against three different strains: *Staphylococcus aureus*, where the operator (+) reflects the presence of antibacterial activity, and the operator (-) reflects the absence of biological action.

| ***Staphylococcus aureus*** | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| DITMBA | Strain | | | IRes | Strain | | | TIPh | Strain | | | RiOL | Strain | | |
| | 1 | 2 | 3 | | 1 | 2 | 3 | | 1 | 2 | 3 | | 1 | 2 | 3 |
| 3.0 мг | + | + | + | 0.4 μгр | + | + | + | 0.6 мг | + | - | - | 0.4 мг | + | + | + |
| 4.0 мг | + | + | + | 0.5 μгр | + | + | + | 1.2 мг | + | + | + | 0.45 мг | + | + | + |
| 6.0 мг | + | + | + | 0.6 μгр | + | + | + | 1.8 мг | + | + | + | 0.5 мг | + | + | + |
| | | | | 0.7 μгр | + | + | + | 2.4 мг | + | + | + | 0.55 мг | + | + | + |
| | | | | 0.8 μгр | + | + | + | | | | | 0.6 мг | + | + | + |

Table 3. Table illustrating the activity of DITMBA, iodoresorcinol (IRes), 2,4,6-triiodophenol (TIPh) and riodoxol (RiOL) against three different strains of *Escherichia coli*, in which the presence of antibacterial activity, and with the operator (-) no biological action is reflected.

| ***Escherichia coli*** | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| DITMBA | Strain | | | IRes | Strain | | | TIPh | Strain | | | RiOL | Strain | | |
| | 1 | 2 | 3 | | 1 | 2 | 3 | | 1 | 2 | 3 | | 1 | 2 | 3 |
| 3.0 мг | + | - | - | 0.4 μгр | + | + | + | 0.6 мг | - | - | - | 0.4 мг | + | + | + |
| 4.0 мг | + | - | + | 0.5 μгр | + | + | + | 1.2 мг | + | + | + | 0.45 мг | + | + | + |
| 6.0 мг | + | + | + | 0.6 μгр | + | + | + | 1.8 мг | + | + | + | 0.5 мг | + | + | + |
| | | | | 0.7 μгр | + | + | + | 2.4 мг | + | + | + | 0.55 мг | + | + | + |
| | | | | 0.8 μгр | + | + | + | | | | | 0.6 мг | + | + | + |

**2. Antimicrobial activity determined according to the classical method of serial dilution (Tables 6, 7, 8 and 9).**

Table 4. Table illustrating the activity of Riodoxol against a selected strain of each of the microorganisms tested. The table with the operator (-) shows the presence of the effect of suppressed microbial growth, and with the operator (+) - the absence of such activity.

| Concentration, µg/mL / Biological species | 300 | 150 | 75 | 37.5 | 18.75 | Positive control |
|---|---|---|---|---|---|---|
| ***Staphylococcus aureus*** | - | - | - | + | + | + |
| ***Escherichia coli*** | - | - | - | + | + | + |
| ***Candida albicans*** | - | - | - | - | + | + |

Table 5. Table illustrating the activity of the substance Iodoresorcinol against a selected strain of each species of microorganisms tested. The table with the operator (-) shows the presence of the effect of suppressed microbial growth, and with the operator (+) - the absence of such activity.

| Concentration, µg/mL / Biological species | 800 | 400 | 200 | 100 | 50 | Positive control |
|---|---|---|---|---|---|---|
| ***Staphylococcus aureus*** | - | - | - | - | - | + |
| ***Escherichia coli*** | - | - | - | - | + | + |
| ***Candida albicans*** | - | - | - | - | + | + |

Table 6. Table illustrating the activity of 2,4,6-triiodophenol against a selected strain of each of the microorganisms tested. The table with the operator (-) shows the presence of the effect of suppressed microbial growth, and with the operator (+) - the absence of such activity

| Concentration, µg/mL / Biological species | 1200 | 600 | 300 | 150 | 75 | Positive control |
|---|---|---|---|---|---|---|
| ***Staphylococcus aureus*** | - | - | - | - | + | + |
| ***Escherichia coli*** | - | - | + | + | + | + |
| ***Candida albicans*** | - | - | - | + | + | + |

Table 7. Table illustrating the activity of DITMBA against a selected strain of each microbial species. The table with the operator (-) shows the presence of the effect of suppressed microbial growth, and with the operator (+) - the absence of such activity.

| Concentration, µg/mL / Biological species | 3000 | 1500 | 750 | 375 | 187.5 | Positive control |
|---|---|---|---|---|---|---|
| ***Staphylococcus aureus*** | - | - | - | + | + | + |
| ***Escherichia coli*** | - | - | + | + | + | + |
| ***Candida albicans*** | + | + | + | + | + | + |

As can be seen from the presented results, the newly synthesized organoiodic compound showed significant activity and a wide range of cid action against all studied bacterial strains of Gram - (+) representatives of *Staphylococcus* and Gram - (-) representatives of *Escherichia*.

Unfortunately, however, this acid did not exhibit the antifungal effect inherent in all other iodine-substituted organic substances, even at significantly higher operating concentrations.

Similar patterns in the biological behavior of DITMBA were established with the classical method of serial dilutions - data that we would use as an indication of the plausibility and veracity of the results obtained by our modified microbiological method; in addition, similar correlations were found in the data on the biological manifestations of all other organoiodic substances.

The significant differences in the activity (antifungal and antibacterial) of DITMBA from those of all other organic iodine substances tested by us (as can be seen from the data in all the above tables) are associated with the peculiarities of molecular symmetry. The latter, which we observe only in DITMBA, can be indicated as the most probable reason for the higher resistance (chemical) of this analyte and the reported higher MIC values of it, to all microorganisms.

Although the observations may be microbiologically negative they can also be used as a clear advantage in the possible study of the potential of the acid in question in the field of imaging and medicine, as an ionic mononuclear ICA simulator.

## VI. CONCLUSIONS

1. A new organoiodic compound, 2,6-diiodo-3,4,5-trimethoxybenzoic acid (DITMBA), has been synthesized

2. The optimal reaction conditions have been established, allowing the synthesis of DITMBA in maximum yield (≥98%) and with extremely high (≥99%) purity.

3. A detailed study of the molecular structure of DITMBA was conducted and the main regularities in its unique spectral behavior were established.

4. The antibacterial activity of DITMBA and its inert behavior with respect to DNA macromolecules was determined.

5. The X-ray contrast potential of DITMBA was assessed.

6. A new protocol for the synthesis of triiodosubstituted aromatic acids, analogues of the pharmacopoeial amidotrizoic acid, has been proposed.

7. Two new *meta*-terphenyl derivatives with a central 3,4,5-trimethoxybenzoic carboxyl motif have been synthesized.

8. The co-crystallizing ability of DITMBA against four N-containing pharmacopoeial agents was determined.

## VII. CONTRIBUTIONS

1. A new organoiodic acid, 2,6-diiodo-3,4,5-trimethoxybenzoic acid (DITMBA), has been synthesized and studied in detail.

2. The influence of *ortho*-positioned iodine atoms on the structural and spectral behavior of the carboxyl DITMBA functional has been established.

2. Two new *meta*-terphenyl acids have been synthesized.

3. A new, effective microbiological method for testing poorly soluble organoiodic compounds has been developed.

4. *Allium cepa* test applied for the first time on an organoiiodic substance.

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