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Separation, analyses and syntheses of trimethoprim impurities

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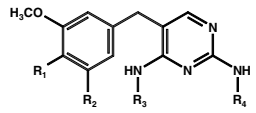
The analysis of several impurities of the chemotherapeutic agent trimethoprim with various methods, including thin layer chromatography, gas chromatography, capillary electrophoresis as well as nuclear magnetic resonance is described. These methods were used to identify new impurities in trimethoprim batches. The main impurities were separated by column chromatography. To ensure the identity of the impurities, *de novo* syntheses were successfully carried out. With the methods described, it was possible to detect, separate and identify new impurities in trimethoprim batches.

1. Introduction

Trimethoprim (**1**) (2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine) is usually administered in combination with sulfamethoxazol ("co-trimoxaol"). It is widely used against grampositive and gramnegative bacteria causing urinary tract, respiratory tract, and gastrointestinal infections. Furthermore, it is also used in AIDS patients to treat infections caused by *Pneumocystis carinii* [1]. The drug is currently synthesized by many different companies.

Due to our observations that trimethoprim batches have been accompanied by new impurities we made efforts to identify these impurities. Therefore, all samples were investigated by different analytical methods such as TLC, HPLC, GC-MS, CE as well as NMR. The main impurities have been separated by column chromatography. To ensure our observations, we additionally synthesized the main impurities of concern.



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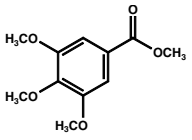
2: $R_1 = OCH_2CH_3$, $R_2 = OCH_3$, $R_3 = R_4 = H$
3: $R_1 = OCH_3$, $R_2 = Br$, $R_3 = R_4 = H$
7: $R_1 = R_2 = OCH_3$, $R_3 = H$, $R_4 = CH_3$
8: $R_1 = R_2 = OCH_3$, $R_3 = CH_3$, $R_4 = H$



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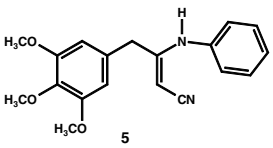

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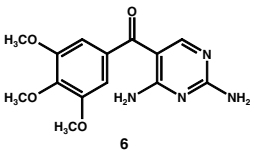

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Development and validation of the HPLC method to quantify the impurities have already been described [2]. This article is focused on the synthesis and analysis of the impurities of trimethoprim.

2. Investigations and results

2.1. Thin layer chromatography

The TLC analyses of 25 industrial batches according to the instructions of the DAB 10 [3], Pharm. Eur. 1997, including supplement 1998, [4] and USP 24 [5] turned out to be insufficient to separate trimethoprim and its impurities. Therefore, two new TLC methods were developed in order to determine the impurities of trimethoprim.

Both methods are described in the experimental section. Typical chromatograms are shown in Fig. 1 (reversed phase method) and Fig. 2 (normal phase method).

As illustrated in Fig. 1, trimethoprim had a retention factor of 0.52, and could therefore clearly be separated from all impurities using the reversed phase method. The retention factor of 2,4-diamino-5-(3-bromo-4,5-dimethoxybenzyl)pyrimidine (**3**) was determined to be 0.29. 3,4,5-Trimethoxybenzoic acid methylate (**4**) and 3-anilino-2-(3,4,5-trimethozybenzyl)acrylonitrile (**5**) showed retention factors of 0.08 and 0.02, respectively. For the identification of these derivatives no further investigations were necessary.

For the definite identification of 2,4-diamino-5-(4-ethoxy-3,5-dimethoxybenzyl)pyrimidine (**2**), 2,4-diamino-5-(3,4,5-trimethoxybenzoyl)pyrimidine (**6**), 4-amino-2-methylamino-5-(3,4,5-trimethoxybenzyl)pyrimidine (**7**) and 2-amino-4-methylamino-5-(3,4,5-trimethoxybenzyl)pyrimidine (**8**) the second, normal phase method was developed. With this method, separation of the impurities **2**, **6**, **7**, and **8** was achieved (see Fig. 2). Trimethoprim (**1**) and the ethoxy derivative **2** were determined to show almost the same retention time. However, with the combination of both methods, the definite identification was possible. 4-Amino-2-methylamino-5-(3,4,5-trimethoxybenzyl)pyrimidine (**7**) with a retention factor of 0.55, and 2,4-diamino-5-(3,4,5-trimethoxy-

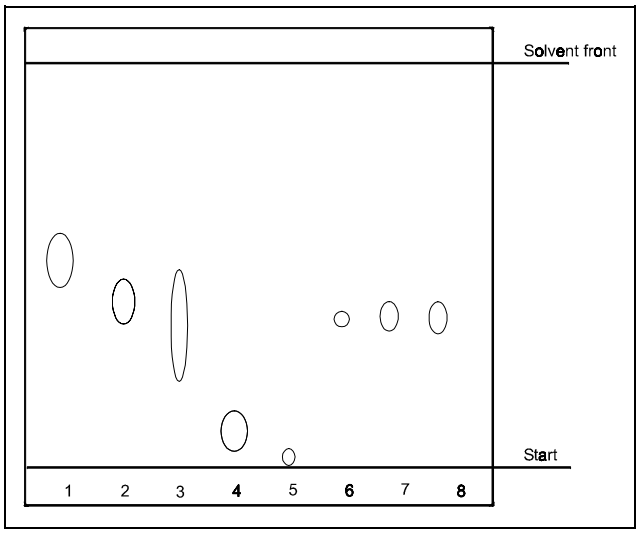


Fig. 1: TLC method 1 (reversed phase method). The chromatogram is showing the retention times of all investigated compounds **1**–**8**

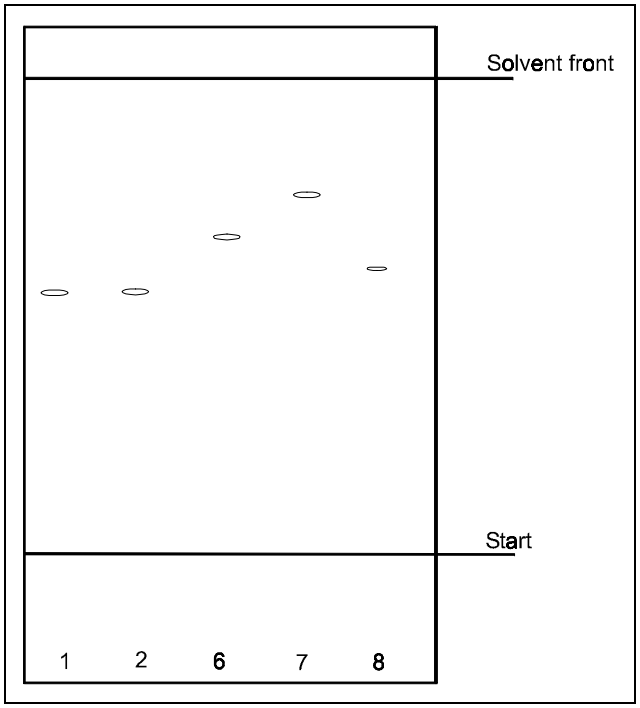


Fig. 2: TLC method 2 (normal phase method). The retention times of trimethoprim (**1**), and impurities **2**, **6**, **7** and **8** are compared

benzoyl)pyrimidine (**6**), with a retention factor of 0.67 could easily be identified with this method as apparent from Fig. 2.

All trimethoprim batches have been investigated with these methods. It turned out that a series of batches showed the ethoxy derivative **2** in concentrations up to 1%. To ensure the identity of this derivative, a column chromatography method was developed to separate impurity **2**.

2.2. Column chromatography

Using a mixture of chloroform and methanol as mobile phase it was possible to separate impurities **2**, **6,** and **7** from trimethoprim (**1**). After the isolation of these derivatives, their properties were investigated and compared with the synthesized ones and proven to be identical. The data are given in the experimental section.

2.3. Gas chromatography and mass spectrometry

GS-MS is usually a very versatile tool to identify impurities in low concentrations. Thus, it was possible to identify at least traces of the impurity **7** in each sample. Furthermore, traces of benzoyl derivative **6** could be detected in concentrations which could not be detected with any other method described. However, the identification of ethoxy derivative **2** was hampered by coelution with trimethoprim under the conditions used. After isolation by column chromatography and parallel synthesis of the ethoxy derivative, it could also be unambiguously identified.

GS-MS gave evidence for traces of compounds **6** and **7**. Compound **8** was not found in any sample.

2.4. Capillary electrophoresis with electrochemical detection

The applicability of capillary electrophoresis (CE) with electrochemical detection was studied for the separation of

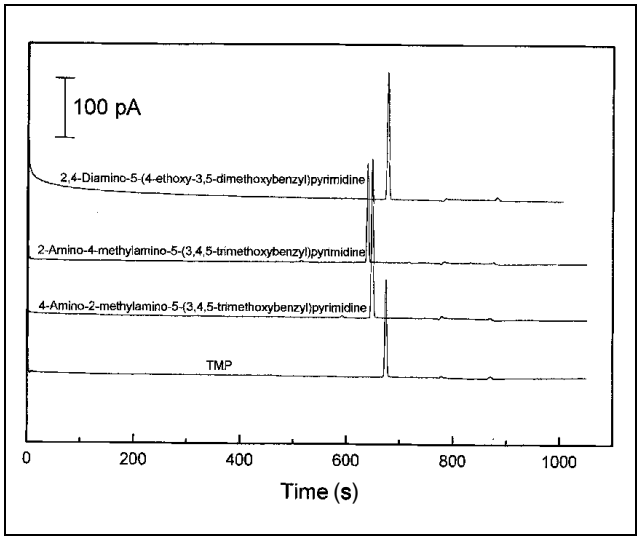


Fig. 3: Capillary electrophoresis with electrochemical detection. Comparison of migration times of trimethoprim (**1**), and impurities **2**, **7** and **8**

trimethoprim (**1**) and impurities **2**, **7** and **8**. As illustrated in Fig. 3, compounds **1**, **7** and **8** could be separated under the experimental conditions used. However, the ethoxy derivative **2** showed the same migration time as trimethoprim (**1**).

To achieve a separation of impurities from the trimethoprim signal the compounds to be separated should exhibit differences regarding the degree of protonation/deprotonation in the non-aqueous buffer, thus forming species with different electrophoretic mobilities in CE measurements.

The advantage of non-aqueous CE with electrochemical detection is that the high sensitivity offered by the amperometric detector can be combined with high stability of electrochemical responses in acetonitrile-based media. In addition, the solubility of trimethoprim (**1**) in the non-aqueous separation buffer is very good which facilitates the investigation of minor impurities. The limits of detection for the impurities were 8 ng/ml (compound **7**) and 6 ng/ml (compound **8**), respectively.

2.5. ^{1}H Nuclear magnetic resonance spectroscopy

^{1}H NMR spectra proved to be a useful tool for the investigations on impurities of the samples. This method was successfully applied to the identification and determination of the following impurities: ethoxy derivative **2**, benzoyl derivative **6** and 2-methylamino derivative **7**.

As shown in Fig. 4, it was possible to detect about 0.1% impurity of these compounds added to a stock solution of 10 mg trimethoprim CRS in 1 ml of $CDCl_3$.

2.6. Syntheses

The syntheses of the compounds were carried out following literature methods [6–13]. Nevertheless, we briefly want to summarize our investigations.

The benzoyl derivative **6** was synthesized by oxidation of trimethoprim **1** with activated manganese dioxide.

Scheme 1 describes the synthesis of the ethoxy derivative **2**. Syringaaldehyde (**9**) was reacted with diethylsulfate yielding the ethoxy aldehyde **10**. After reaction with 3-ethoxypropionitrile, the intermediate was condensed with guanidine carbonate forming **2**.

To synthesize the isomers **7** and **8**, two different approaches have been carried out. The syntheses are pre-

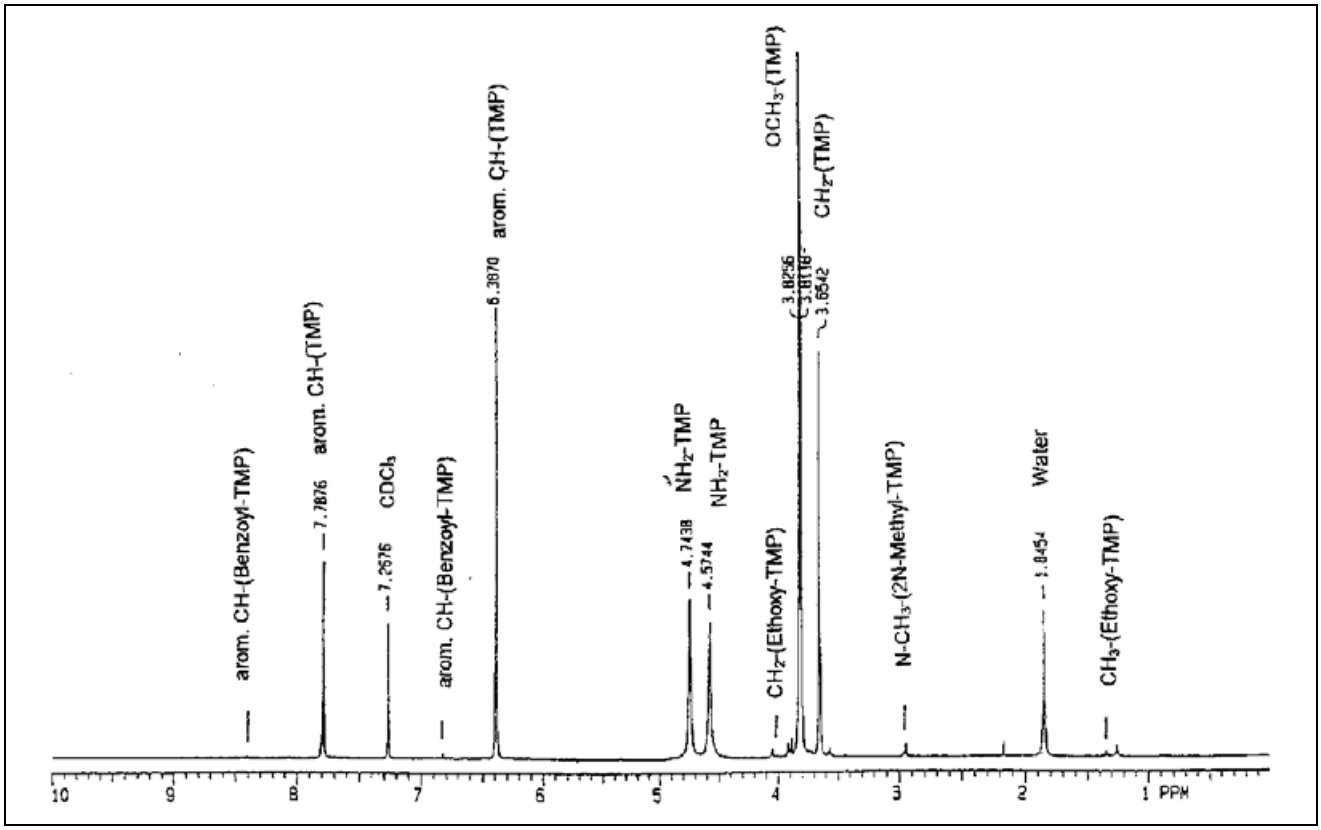
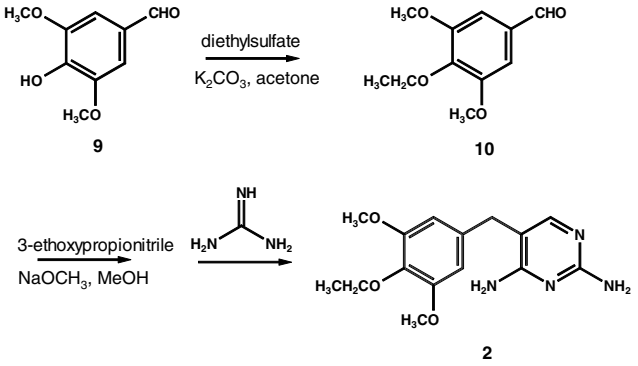
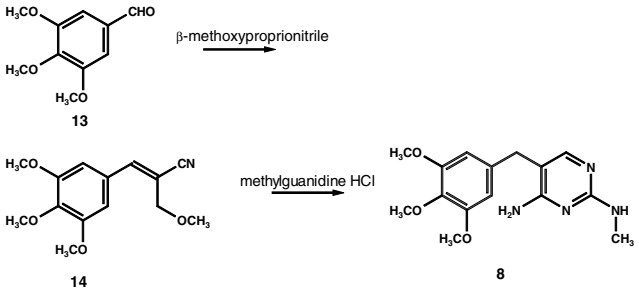


Fig. 4: ^{1}H nuclear magnetic resonance spectrum. Trimethoprim (TMP) spiked with Ethoxy TMP **2**, Benzoyl TMP **6**, and 2N-Methyl TMP **7**

Scheme 1



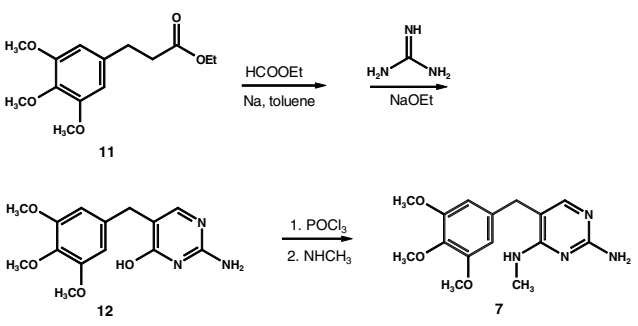
Scheme 3



sented in Schemes 2 and 3. In order to prepare **7**, trimethoxypropionic acid ethylate (**11**) was reacted with formic acid ethylate, followed by treatment with guanidine carbonate. The resulting hydroxy derivative **12** was subsequently reacted with phosphoryl chloride and methylamine to form **7**.

Analogously to trimethoprim itself, **8** was prepared using trimethoxybenzaldehyde (**13**) as starting material. Reaction with β-methoxypropionitrile yielded the intermediate **14**, the subsequent reaction with methylguanidine formed **8**.

Scheme 2



3. Discussion

With the combination of all the methods described herein, we were able to identify and quantify new impurities in trimethoprim batches. As a consequence of our investigations, the European Monograph of trimethoprim has recently been revised [14].

Especially TLC has been demonstrated to be a fast, powerful and simple separation method for structurally similar compounds. Additionally, ^{1}H NMR gave important hints for the identity of the impurities. The lower detection limit of GC-MS and CE allowed additional statements of traces of impurities that could not be detected with TLC or ^{1}H NMR. A very good agreement has been

found between the impurity profiles determined by the methods described.
The synthesized compounds and those isolated by column chromatography have been shown to be identical. The work presented here underlines once more the importance of updating analytical procedures of pharmaceutical compounds.

4. Experimental

4.1. Materials and reagents and equipment

The material and reagents for the analytical investigations were of analytical grade and were obtained from Merck (Darmstadt, Germany). Highly purified silica gel, particle size range 63–200 μm, also obtained from Merck, was used for column chromatography. Impurities 2,4-diamino-5-(3-bromo-4,5-dimethoxybenzyl)pyrimidine (**3**) and 3-anilino-2-(3,4,5-trimethoxybenzyl)acrylonitrile (**5**) were obtained from the Bundesinstitut für Arzneimittel und Medizinprodukte (Federal Institute for Drugs and Medical Devices), Berlin, Germany. GC-MS, CE and TLC are described below in detail. All other materials and reagents were obtained from commercial suppliers and used without further purification.
Melting points were determined in a Büchi 535 apparatus, or a Gallenkamp Variable Heater, respectively, and are uncorrected. UV spectra were recorded on a Shimadzu UV VIS photometer. The concentration of the solution was 5 μg/ml. The compounds were dissolved in a mixture of 3 volumes of methanol and 7 volumes of an aqueous solution of 1.4 g/l sodium perchlorate monohydrate. The mixture was adjusted with conc. phosphoric acid to pH 3.4.
IR spectra were recorded on a Perkin Elmer FT-IR PC 16 (Perkin Elmer, Überlingen, Germany) with KBr disks. ^{1}H NMR spectra were recorded on a Varian Gemini 300 spectrometer (^{1}H: 300 MHz). Chemical shifts are given in ppm (δ). The chemical shifts of the remaining protons of the deuterated solvents served as internal standard δ (^{1}H, $CDCl_3 = 7.24$)
Elemental analyses for C, H and N were determined at the Institute of Organic Chemistry, Leipzig, Germany, and were within ±0.4% of the theoretical values. The compounds were prepared following literature procedures. Compound **8** has not yet been described.

4.2. Analytical procedures

4.2.1. Thin layer chromatography

Method 1 (reversed phase method):
HPTLC plates, covered with RP-18 $WF_{254\ s}$, 10 × 10 cm were used as stationary phase. As mobile phase a mixture of a solution of 0.1 M tetramethylammoniumchloride, methanol and formic acid (70 : 30 : 2, v/v) was used. The chamber was allowed to saturate before development of the chromatograms. 0.5 mg of each compound were dissolved in 10 ml of methanol, 200 μg were applied. The chromatograms were developed over 60 min. The solvent front was 8.5 cm. The spots on the chromatograms were detected with UV at 254 nm.
Method 2 (normal phase method):
HPTLC plates with concentration zones, silica 60 F 254, 10 × 10 cm were used. A mixture of chloroform, methanol and conc. ammonia (25%) was used as mobile phase (90 : 15 : 1). The chamber was allowed to saturate before development of the chromatograms. A solution of 0.5 mg of the compounds in 10 ml of methanol was prepared, 200 μg were applied. The chromatograms were developed within 15 min. The spots on the chromatograms were detected at 254 nm.

4.2.2. Column chromatography

As stationary phase, silica 60, was used for column chromatography. The size of the column was 200 × 22 mm. The mobile phase was composed of chloroform and methanol (100 : 5, v/v). 100 mg impure trimethoprim was used for each separation. The flow rate was about 50–60 drops per min. Fractions were collected; the fraction size was 2 ml. HPTLC plates following method B checked the separation.

4.2.3. Gas chromatography and mass spectrometry

The GC-MS instrument (Hewlett-Packard 5890/II-5971) was equipped with a fused-silica capillary column ULTRA 1 (25 m × 0.2 mm × 0.33 μm). Helium was used as the carrier gas (1 ml · min^{-1}). The injection port of the GC was kept at 250 °C. Generally, the splitless mode of injection (1 μl) was used. MS were recorded in the electron impact ionization mode at 70 eV. The temperature program was as follows: 150 °C to 300 °C at 20° × min^{-1} after 1 min at 150 °C and 3 min at 300 °C, respectively.

4.2.4. Capillary electrophoresis with electrochemical detection

The separations were carried out using a laboratory-made CE system with electrochemical detection. The high-voltage power supply (Model HCN 7E-35000, F. u. G. Elektronik, Rosenheim-Langenpfunzen, Germany) was capable of delivering 0–35 kV. The high-voltage input of the capillary system was housed in a Plexiglass box fitted with a safety interlock switch. The electrochemical detector was based on an end-column arrangement of a 25-μm platinum microdisk electrode and a 75-μm I.D. capillary. Fused silica capillaries with I.D. of 75 μm and O.D. of 360 μm were obtained from Photonetics GmbH (Kehl, Germany). For the measurements described a capillary with a length of 95 cm was used. The separation buffer consisted of a solution of acetonitrile containing 1 M acetic acid and 10 mM sodium acetate. A separation voltage of 20 kV was applied and sample injection was done electrokinetically (20 s at 5 kV). Potential control of the electrochemical detector as well as data acquisition was accomplished by means of the electrochemical measuring system Autolab PGSTAT 10 (Eco Chemie, Utrecht, The Netherlands) equipped with a low-current amplifier module. The detection potential was 1.7 V vs. a silver/silver chloride reference electrode.

4.2.5. ^{1}H Nuclear magnetic resonance spectrocospy

1H NMR spectra were determined on a Varian Gemini 300 spectrometer (^{1}H: 300 MHz) using $CDCl_3 = 7.24$. The number of pulses was 500.

4.3. Syntheses

4.3.1. 2,4-Diamino-5-(3,5-dimethoxy-4-ethoxybenzyl)pyrimidine (**2**) [6]

A mixture of 10.5 g (0.05 mol) **10** and 5.4 g (0.1 mol) sodium methanolate, 5.0 g (0.05 mol) 3-ethoxypropionitrile, and 20.0 g powdered molecular sieve 3 Å in 150 ml of methanol were refluxed for 4 h. The precipitate was filtered off. To the filtrate, 24.0 g (0.15 mol) guanidine carbonate and 10.8 g (0.2 mol) sodium methanolate in 100 ml of methanol were added and refluxed over 48 h. After cooling, ice was added and the solution was allowed to precipitate over night. The precipitate was dissolved in methanol, filtered off and frozen at −20 °C for 48 h. The precipitate was filtered off and dried *in vacuo*.
Yield: 5.3 g (35%); M.p..: 182–185 °C (Lit. 185–187 °C); UV: 271 (0.150); IR: 3458, 3314, 3156, 2972, 1632, 1597, 1559; ^{1}H NMR ($CDCl_3$) δ 1.35 (t, 3 H, $CH_2\underline{CH_3}$), 3.65 (s, 2 H, CH_2), 3.81 and 3.83 (2 × s, 9 H, OCH_3), 4,05 (d, 2 H, $\underline{CH_2}CH_3$), 4.57 (s, 2 H, NH_2), 6.39 (s, 2 H, $H_{arom.}$), 7.78 (s, 1H, $H_{pyrimidine}$).

4.3.2. 2,4-Diamino-5-(3,4,5-trimethoxybenzoyl)pyrimidine (**6**) [7]

5.8 g (0.02 mol) Trimethoprim (**1**) and 17.2 g (0.2 mol) activated manganese dioxide were grated in a mortar. The mixture was heated under stirring for 8 h at 120 °C. After cooling, a mixture of 150 ml of ethyl acetate and methanol (8 : 2, v:v) was added, stirred and filtered off. The solvent was removed *in vacuo*, and the residue was chromatographed on silica gel using ethyl acetate/methanol (8 : 2, v : v).
Yield: 4.9 g (71%); M.p.: 193–195 °C (Lit. 197–198 °C); UV: 227 (0.284), 255 (0.161), 298 (0.130); IR: 3387, 3136, 2937, 1675, 1581, 1503; ^{1}H NMR ($CDCl_3$) δ 3.89 and 3.92 (2 × s, 9 H, OCH_3), 5.22 (s, 2 H, NH_2, exchange with MeOD), 4.55 (s, 2 H, NH_2, exchange with MeOD), 6.83 (s, 2 H, $H_{arom.}$), 8.41 (s, 1 H, $H_{pyrimidine}$).

4.3.3. 2-Amino-4-methylamino-(3,4,5-trimethoxybenzyl)pyrimidine (**7**) [8]

A solution of 5.82 g (0.02 mol) **12** and 50 ml of phosphoryl chloride was refluxed for 8 h. After cooling, the solution was cautiously hydrolyzed. The precipitate was filtered off. The filtrate was neutralized using 25% ammonia solution. The precipitate was filtered off. The raw product (2-amino-4-chloro-5-(3,4,5-trimethoxybenzyl)-pyrimidine) was immediately reacted with 50 ml of 33% methylamine in ethanolic solution and stirred for 48 h. After removing the solvents *in vacuo*, the residue was chromatographed on silica using a mixture of ethylacetate : methanol (8 : 2, v:v).
Yield: 3.0 g (50%); M.p.: 165–167 °C (Lit. 168–169 °C); UV: 240 (0.275), 280 (0.084); IR: 3340, 3169, 2937, 1603, 1506; ^{1}H NMR ($CDCl_3$) δ 2.87 (d, 3 H, NCH_3, exchange with MeOD: s), 3.60 (s, 2 H, CH_2), 3.81 and 3.83 (2 × s, 9 H, OCH_3), 4.40 (m, 1 H, $N\underline{H}CH_3$, exchange with MeOD), 4.76 (s, 2 H, NH_2, exchange with MeOD), 6.36 (s, 2 H, $H_{arom.}$), 7.65 (s, 1 H, $CH_{pyrimidine}$).

4.3.4. 4-Amino-2-methylamino-(3,4,5-trimethoxybenzyl)pyrimidine (**8**) [9–11, 13]

A mixture of 2.08 g (19 mmol) methylguanidine hydrochloride and 1.13 g (20.9 mmol) sodium methanolate was stirred for 5 d in methanol. At the fifth day, a second mixture of 1.0 g (3.8 mmol) **14** and 0.42 g (7.8 mmol) in methanol was refluxed for 24 h. Both mixtures were combined at the sixth day and refluxed for another 24 h. After cooling, the solvents were removed *in vacuo*, and the residue was dissolved in a mixture of chloroform and water (1 : 1, v : v). The water layer was extracted twice with chloroform. The combined organic layers were washed with water, dried over sodium sulfate and the solvent was removed *in vacuo*.

The residue was chromatographed on silica (45 cm × 2 cm) with a mixture of chloroform and methanol. The remaining oil was dissolved in ethyl acetate and allowed to crystallize overnight. The crystals were collected and dried.
Yield: 0.21 g (18%); M.p.: 159–160.5 °C; UV: 280 (0.070); IR: 3469, 3342, 3258, 1640, 1595, 1508; ^{1}H NMR ($CDCl_3$) δ 2.96 (d, 3 H, NCH_3, exchange with MeOD: s), 3.65 (s, 2 H, CH_2), 3.82 and 3.83 (2 × s, 9 H, OCH_3), 4.48 (s, 2 H, NH_2, exchange with MeOD); 4.73 (m, 1 H, $N\underline{H}CH_3$, exchange with MeOD), 6.40 (s, 2 H, $H_{arom.}$), 7.81 (s, 1 H, $CH_{pyrimidine}$).

4.3.5. 3,5-Dimethoxy-4-ethoxybenzaldehyde (**10**) [12]

A mixture of 5.5 g (0.03 mol) syringaaldehyde (**9**), 15.2 g (0.11 mol) potassium carbonate, and 15.4 g (0.1 mol) diethylsulfate in 100 ml of acetone were stirred for 72 h at 50 °C. The precipitate was filtered off. The solvent of the filtrate was removed *in vacuo*, and the residue was chromatographed on silica using a mixture of ethyl acetate:n-hexane (1 : 1, v:v).
Yield: 2.5 g (40%); M.p.: 46–47 °C (Lit. 49–51 °C).

4.3.6. 2-Amino-4-hydroxy-5-(3,4,5-trimethoxybenzyl)pyrimidine (**12**) [8]

To 4 ml (0.055 mol) of a suspension of 30% sodium in toluene, a solution of 12.5 g (0.05 mol) 3-(3,4,5-trimethoxyphenyl)propionic acid ethylate (**11**) and 3.7 g formic acid ethylate in 50 ml of diethyl ether were added at 0 °C and stirred for 24 h.
The precipitate was filtered off and added to a mixture of 24 g (0.15 mol) guanidine carbonate and 10.8 g (0.2 mol) sodium methanolate in 100 ml methanol. The mixture was refluxed for 48 h. After cooling, the mixture was poured onto ice, conc. hydrochloric acid was added. The solution was neutralized with 25% ammonia solution and allowed to precipitate overnight. The precipitate was filtered off and dried.
Yield: 4.1 g (30%); M.p.: 252–255 °C.

4.3.7. 2-Methoxymethylene-3-(3,4,5-trimethoxyphenyl)acrylonitrile (**14**) [11]

To a mixture of 8.1 g (150 mmol) sodium methanolate in 20 ml of methanol, a mixture of 9.81 g (50 mmol) trimethoxybenzaldehyde (**13**) und 13 ml of β-methoxypropionitrile dissolved in 13.5 ml of methanol were added within 1 h under ice cooling. The temperature of the mixture is not allowed to exceed 10 °C. The mixture was stirred for 24 h at RT, and then cooled for 12 h at −15 °C. The precipitate was filtered off, washed three times with 20 ml of water and three times with 20 ml of 75% methanol.
Yield: 5.8 g (44%)

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