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An aza-Michael addition product causes incompatibility between etacrynic acid and theophylline in a paediatric cardiological ICU

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In a compatibility study of parenteral drugs commonly used in paediatric cardiological intensive care units, an unknown reaction product was found in a mixture of etacrynic acid and theophylline. The conditions in terms of the concentration of etacrynic acid and theophylline as well as the materials used corresponded to the conditions in the intensive care unit. Initially, the reaction product appeared as a significant and increasing peak in the chromatograms when determining the content of etacrynic acid and theophylline via HPLC. At the same time, the concentrations of both drugs decreased. A literature search in the chemical databases Reaxys® and Scifinder® revealed a patent from 1967 describing an aza-Michael addition between etacrynic acid and theophylline to either N-7 or N-9. Using LC-MS/MS experiments, we were able to confirm that Michael-like reaction between etacrynic acid and theophylline occurs. To elucidate the exact structure of the reaction product we performed NMR experiments (COSY, HSQC and HMBC). With the acquired data we were finally able to identify the unknown compound as the N-7 substituted adduct [2-(2,3-dichloro-4-{2-[(1,3-dimethyl-2,6-dioxo-2,3-dihydro-1*H*-purin-7(6*H*)-yl)methyl]butanoyl}phenoxy)acetic acid]. Our findings show that etacrynic acid and theophylline should not be mixed and should be administered through separate venous lines when infused.

1. Introduction

Patients in intensive care units (ICU) often receive a variety of different parenteral drugs. However, the number of central or peripheral venous accesses is limited, especially in critically ill children. Ideally, compatibility studies are available to help healthcare professionals decide which drugs can be administered together and which are better administered alone. But this is not always the case, as the drugs and concentrations can vary greatly from ward to ward and patient to patient. For example, the loop diuretic etacrynic acid (Eca; Fig. 1a), first described by Schultz et al. (1962), is rarely used today due to potential side effects (Alisky and Tuttle 2003). Nevertheless, in paediatric cardiological intensive care units, etacrynic acid is still used in addition to or as an alternative to other diuretics, e.g. furosemide, when these alone are not sufficiently effective (Kim et al. 1971; Ricci et al. 2015). Etacrynic acid can react as a Michael acceptor due to the a,ß-unsaturated carbonyl group with nucleophilic compounds (Michael donor). Another drug used in paediatric cardiological intensive care is theophylline (Tph; Fig. 1b), which is a bronchodilator and is usually used to treat asthma and chronic obstructive pulmonary disease (COPD). Additionally, a significant diuretic effect has also been observed, especially in critically ill children (Da Silva et al. 2012; Bell et al. 1998). Reactions of theophylline as a nucleophile are mainly observed at N-7 of the purine skeleton, very seldom reactions at positions N-9 or C-8 have been reported (Lister 1979). This correlates well with the predominance of the 7-H tautomer versus the 9-H tautomer and the higher basicity of N-7 compared with N-9 (Gulevskaya and Pozharskii 1991; Reisch et al. 1994). The aza-Michael type addition of theophylline to electron deficient alkenes is also a known reaction with synthetic potential (Rybár and Štibrányi 1973; Horváth 1995). Recently, the addition of aromatic amines, which have similar basicity compared with theophylline to enones in aqueos solutions have been reported (Dutt et al. 2020).

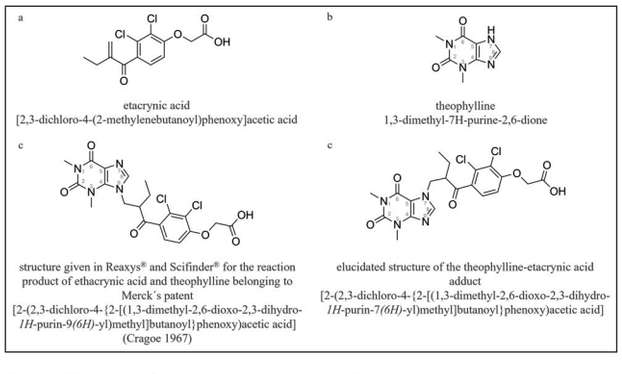

Fig. 1: Formulas of etacrynic acid and theophylline and the two adducts

As part of a compatibility study of parenteral drugs used in a paediatric cardiological intensive care unit, an unknown reaction product between etacrynic acid contained in the drug Reomax® and theophylline contained in the drug afpred® forte-THEO was identified and finally, the structure was completely elucidated using LC-MS/MS and NMR experiments.

2. Investigations and results

2.1. Method validation for quantification

For quantification of the drugs, an HPLC method was developed. The results of the validation of the HPLC method are presented in Table 1. Tph and Eca were quantified in the range of 0.01 – 0.6 mg/ml with sufficient reproducibility and recovery, therefore the HPLC method was considered appropriate.

Table 1: Results of the validation of the HPLC method

Drug	λ (nm)	Linearity (mg/ml)	t_R (min)	R^2	Equation regression line	max. CV (%)	Recovery* min. – max. (%)
Tph	254	0.01 – 0.6	4.6	1.0000	y = 8614302.6x + 281.4	1.6	98.6 – 100.9
	270			0.9999	y = 18121856.4x + 303	Ø 0.7 ± 0.4	Ø 99.5 ± 0.5
Eca	254	0.01 – 0.6	18.8	1.0000	y = 3851626.1x + 649.4	1.9	97.9 – 101.3
	270			1.0000	y = 4136889.8x + 447.8	Ø 0.9 ± 0.6	Ø 99.1 ± 0.8

λ: wavelength; t_R: mean retention time; R^2 correlation coefficient; CV: coefficient of variation; Eca: ethacrynic acid; Tph: theophylline; Ø: mean ± sd; *includes both the intraday and interday recovery after freezing and thawing

Table 2: pH Values of the different drug combinations measured directly after mixing (start) and after 2 h, 4 h, 24 h, 96 h; Eca: etacrynic acid; Tph: theophylline

combination Eca + Tph (mg/ml)		Start	after 2 h	after 4 h	after 24 h	after 96 h
0.4 + 2	duplicate 1/2	9.17	9.20	9.24	9.28	9.21
	duplicate 2/2	9.15	9.18	9.22	9.30	9.17
0.4 + 0.4	duplicate 1/2	8.86	8.98	8.97	9.13	8.67
	duplicate 2/2	8.97	8.97	8.99	9.11	8.70
0.05 + 2	duplicate 1/2	9.13	9.05	9.09	9.12	9.10
	duplicate 2/2	9.15	9.15	9.16	9.16	9.16
0.05 + 0.4	duplicate 1/2	9.09	9.06	9.04	9.11	9.04
	duplicate 2/2	9.02	8.98	9.03	9.04	9.04

Table 3: Drug concentrations for etacrynic acid (Eca) and theophylline (Tph) relative to the initial content of the different drug combinations measured immediately after mixing and after 2 h, 4 h, 24 h, 96 h

Combination (mg/ml)		Start (%)	After 2 h (%)	After 4 h (%)	After 24 h (%)	After 96 h (%)
0.4	**Eca**	100	77.4 ± 4.6	49.2 ± 2.6	14.1 ± 0.1 *	13.2 ± 0.3 *
2	**Tph**	100	97.1 ± 0.9	95.8 ± 0.9	92.2 ± 0.4	91.0 ± 0.3
0.4	**Eca**	100	94.0 ± 1.4	87.0 ± 0.3	56.3 ± 2.7	42.8 ± 2.3
0.4	**Tph**	100	96.4 ± 1.5	92.5 ± 0.1	73.8 ± 2.7	67.3 ± 0.1
0.05	**Eca**	100	65.6 ± 7.1	45.2 ± 3.6	8.4 ± 1.5 *	6.6 ± 0.1 *
2	**Tph**	100	100.7 ± 0.6	100.2 ± 0.6	101.6 ± 0.7	100.1 ± 0.3
0.05	**Eca**	100	92.7 ± 1.3	79.1 ± 0.0	29.4 ± 0.3 *	13.5 ± 0.1 *
0.4	**Tph**	100	100.0 ± 0.9	99.8 ± 1.4	97.0 ± 0.8	96.5 ± 1.2

*values are extrapolated, since the concentration is outside the calibration (mean ± sd, n = 2^3)

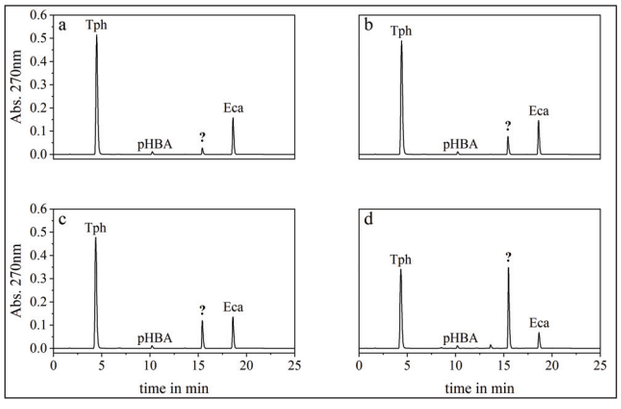

Fig. 2: Chromatogram of the combination of 0.8 mg/ml etacrynic acid (Eca) and 0.8 mg/ml theophylline (Tph); a: immediately after mixing; b: after 2 h; c: after 4 h; d: after 96 h; pHBA: p-hydroxybenzoic acid (an excipient in Remoax); ?: unknown substance

2.2. pH Values

As part of the compatibility study, the pH value of the individual drug solutions as well as the mixtures was routinely measured. The pH of the diluted Reomax® solution was 7.55 for 0.1 mg/ml and 7.58 for 0.8 mg/ml. A pH of 9.04 was measured for the 0.8 mg/ml concentrated afpred® forte-THEO solution and a pH of 9.18 for 4 mg/ml. Table 2 shows the measured pH values of the drug combinations. The pH value is around 9 for all combinations and over the entire storage time.

2.3. High performance liquid chromatography

Figure 2 shows representative chromatograms for the measurement of a mixture of etacrynic acid and theophylline. Immediately after mixing a prominent, additional peak with a retention time of 15.4 minutes is visible, which becomes larger upon prolonged storage. Table 3 shows the concentrations of Eca and Tph in different combinations. The initial concentration directly after mixing was defined as 100 %. The subsequent measurements after a certain time of storage (2 h, 4 h, 24 h, or 96 h) are shown in relation to the initial content in percent. It can be seen that the concentrations, especially of Eca, which is less concentrated than Thp, decrease continuously. Especially with large excess of Thp, the content of Eca decreases very quickly. For example, the concentration of Eca in combination with the concentrations 0.05 mg/ml Eca and 2 mg/ml Tph drops to 65.6 % after only two hours.

2.4. LC-MS

In a first LC-MS run, besides the molecular ions belonging to etacrynic acid (*m/z* 303) and theophylline (*m/z* 181), another $[M+H]^+$ molecular ion (*m/z* 483) with a typical isotope pattern for two chloro substituents were found. Only one isomer with *m/z* 483 was detected in all LC-MS runs, even with extensive overload, which is shown in the total ion chromatogram in Fig. 3. The

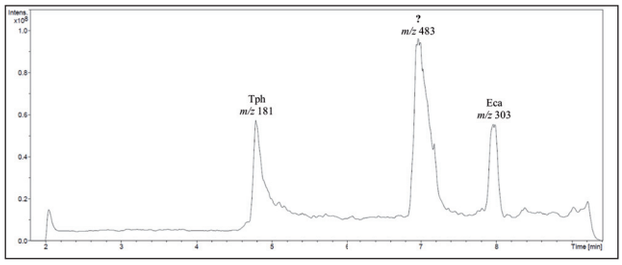

Fig. 3: Representative total ion chromatogram of a mixture of theophylline (Tph) and etacrynic acid (Eca); ?: unknown substance

unknown substance with *m/z* 483 elutes after 7.0 min. This peak was further investigated by MS/MS experiments.

Figure 4 represents the spectrum and the presumed fragments of an ESI MS/MS experiment for the selected ion with *m/z* 483 ± 2. In total, seven fragments were detected. Again, four ions with *m/z* 303, *m/z* 247, *m/z* 227 and *m/z* 189 show the partial isotopic signal for two chloro substituents.

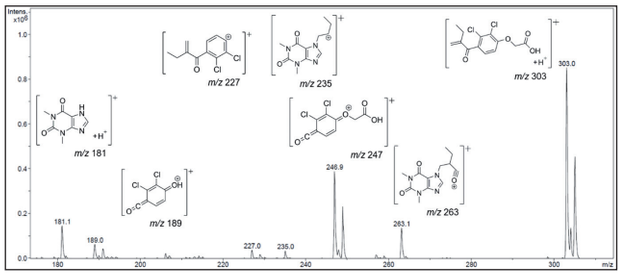

Fig. 4: MS/MS spectrum and presumed ions of the unknown substance with *m/z* 483 ± 2

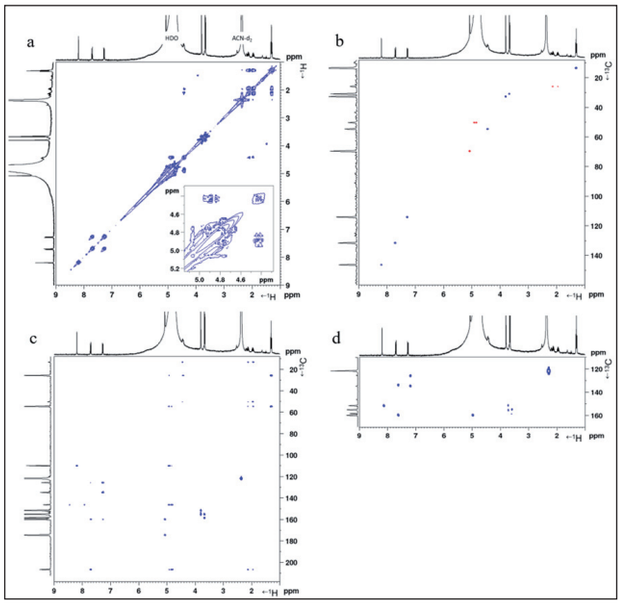

Fig. 5: NMR correlation spectra of the isolated substance a: COSY (HDO: water signal, ACN-d_3: acetonitrile signal); b: HSQC; c: HMBC optimized for 6.2 Hz couplings; d: HMBC optimized for 12.8 Hz couplings; enlarged

2.5. Literature research

Chemical structure search with Reaxys® (Beilstein Registry Number 1195790) and Scifinder® (Chemical Abstracts Number 32893-79-1) revealed a Merck patent from 1967 (Cragoe 1967) showing that a reaction product of Tph and Eca is formed via an aza-Michael addition. The proposed structure was either the N-7- or N-9-substituted isomer. However, only the results of an elemental analysis and no exact structural analysis were published. In Reaxys® (Beilstein registration number 1195790) and Scifinder® (Chemical Abstracts number 32893-79-1) only the N-9 substituted derivative is reported (Cragoe 1967). See Fig. 1c.

2.6. Nuclear magnetic resonance spectroscopy

The unknown substance was isolated and measured with NMR. The sample amount after isolation determined with ERETIC was 0.3 mg in 0.45 ml of a mixture of D_2O and deuterated acetonitrile (1:1). Figure 5 shows the NMR correlation spectra. All ^1H and ^{13}C signals were determined: ^1H NMR (400 MHz, *d*/ppm, D_2O/CD_3CN): 1.30 (t, J = 7.3 Hz, 3 H, CH_2-C$\underline{H}_3$), 1.94 (m, 1 H, C$\underline{H}_2$-CH_3), 2.14 (m, 1 H, C$\underline{H}_2$-CH_3), 3.67 (s, 3 H, N1-CH_3), 3.80 (s, 3 H, N3-CH_3), 4.44 (m, 1 H, CH), 4.84 (m, 1 H, N7-CH_2), 4.92 (m, 1 H, N7-CH_2), 5.07 (s, 2 H, OCH_2), 7.28 (d, J = 8.9 Hz, 1 H, aryl H-5), 7.70 (d, J = 8.9 Hz, 1 H, aryl-H6), 8.16 (s, 1 H, hetaryl-CH), CO_2H not observed due to exchange. ^{13}C NMR (100 MHz, *d*/ppm, D_2O/CD_3CN): 13.4 ($CH_2\underline{C}H_3$), 25.9 ($\underline{C}H_2CH_3$), 30.9 (N1-CH_3), 32.7 (N3-CH_3), 50.3 (N7-CH_2), 54.7 (CH), 69.7 (OCH_2), 110.0 (hetaryl-C5), 114.0 (aryl-C6), 125.7 (aryl-C2), 131.7 (aryl-C5), 133.9 (aryl-C3), 134.9 (aryl-C4), 146.4 (hetaryl-C8), 151.7 (hetaryl-C4), 155.2 (hetaryl-C2), 158.5 (hetaryl-C6), 159.9 (aryl-C1), 174.6 (CO_2H), 206.7 (C=O).

3. Discussion

3.1. Identification of the reaction product

In this study, we identified a reaction product between etacrynic acid and theophylline that has not previously been described under conditions that may occur in an intensive care unit. Fig. 1d represents the identified molecule.

Various degradation products are described for Eca in aqueous solutions. These include a dimer formed after a Diels-Alder-like condensation, a hydrate and another dimer after formaldehyde elimination of the hydrate (Cohen 1971; Görlitzer and Höbbel 1979; Yarwood et al. 1987). However, the expected molecular ions of these degradation products (*m/z* 483 are *m/z* 605, *m/z* 335 and *m/z* 593, respectively) were not observed. In Merck's patent (Cragoe 1967), an aza-Michael addition between Eca and Tph was described. This reaction takes place in water at a pH of 7.1, 25 °C and over a period of 16 h (Cragoe 1967). At this point, it was already very likely that the compound we found was one of the products described in the patent, especially considering that the mixtures of the two drugs Reomax® and afpred® forte-THEO have a pH value of about 9. An alkaline pH favours deprotonation at N-7 of Tph, so that the aza-Michael addition can proceed more easily. A MS/MS experiment then provided further evidence for a Michael-like addition. In particular, the fragment *m/z* 263 confirms this. To elucidate the exact chemical structure, NMR experiments were carried out. Using a HH-COSY, with water suppression technique, all proton signals were evaluated and identified (Fig. 5a) (Berger and Braun 2011). In addition, all ^{13}C carbon signals were determined with two-dimensional heteronuclear correlations, namely a standard HSQC (Fig. 5b) (Berger and Braun 2011) and two HMBC (Fig. 5c–d) experiments (one optimized for 6.2 Hz, and another for 12.8 Hz couplings) (Berger and Braun 2011). The proton spectrum confirmed the formation of the adduct by a Michael-type addition to etacrynic acid. The olefinic protons of the etacrynic acid disappeared, instead a complex multiplet pattern was observed for the diastereotopic protons of the two methylene groups as a chiral centre is generated by the addition reaction. The chemical shifts of the newly formed methylene group (^1H: *d* 4.84 ppm and 4.92 ppm, ^{13}C: *d* 50.3 ppm) and long range correlations of this methylene group with two carbon atoms of the fused imidazole ring of theophylline, one being C-8, the other C-5, only allow the conclusion that it must be the N-7 adduct.

3.2. Conclusion

We were able to clearly identify the reaction product found in a compatibility study of etacrynic acid and theophylline as [2-(2,3-dichloro-4-{2-[(1,3-dimethyl-2,6-dioxo-2,3-dihydro-1*H*-purin-7(6*H*)-yl)methyl]butanoyl}phenoxy)acetic acid]. The reaction conditions, such as the selection of drug concentrations and

the materials and solutions used, were fully adapted to the conditions in a paediatric cardiological ICU. Therefore, etacrynic acid and theophylline should not be infused simultaneously through the same lumen of a venous access.

4. Experimental

4.1. Chemicals and reagents

The drugs Reomax® 50 mg/20 ml vials iv (batch: 079178) and afpred® forte-THEO 200 mg (batch: 90371; 80641) as well as 50 ml polypropylene syringes (Original Perfusor® B. Braun Melsungen AG or BD™ Medical™ Plastipak™) and sterile 0.9 % saline solution (B. Braun Melsungen AG or Fresenius Kabi) were kindly provided by the hospital pharmacy of the UKSH Campus Kiel. All solutions were within the stated expiry time at the time of testing. LC-MS grade water was obtained from VWR (83645.320); acetonitrile gradient grade 99.9 % from honeywell (34851) and sodium dihydrogen phosphate as well as the ready-to-use buffer solutions ROTI®Calipure from Carl Roth (K300.2, A517.1, P713.1, 8086.4). Acetic acid Suprapur® and sodium 3-trimethylsilyl propane sulfonate were obtained from Sigma-Aldrich (1.00066, 178837). The deuterated water 99.9 % (00506) and acetonitrile 99.8 % (00205) were purchased from DEUTERO GmbH.

4.2. Sample preparation

For all test solutions, the drugs Reomax®, which contains etacrynic acid, and afpred® forte-THEO, which contains theophylline, were used. Reomax® was reconstituted with 20 ml of 5 % glucose solution according to the manufacturer's instructions and then diluted with 0.9 % saline to a final concentration of 0.1 mg/ml or 0.8 mg/ml. The drug afpred® forte-THEO was diluted with 0.9 % sodium chloride solution to a concentration of 0.8 mg/ml and 4 mg/ml, respectively. These concentrations correspond to the procedure in paediatric cardiological intensive care. To simulate the concurrent flow through one catheter lumen, the solutions were mixed in 50 ml syringes at a ratio of 1:1. The resulting mixtures have the concentrations 0.4 mg/ml Eca + 2 mg/ml Tph; 0.4 mg/ml Eca + 0.4 mg/ml Tph; 0.05 mg/ml Eca + 2 mg/ml Tph; 0.05 mg/ml Eca + 0.4 mg/ml. The syringes were sealed with a combi-stopper (B. Braun) and stored at room temperature and under laboratory light. Each mixture was prepared in duplicate. Samples were taken immediately after mixing, as well as after 2 h, 4 h, 24 h and 96 h. The pH values of the solutions were determined. Samples were frozen in liquid nitrogen and stored at -80 °C. The samples were freshly thawed before HPLC measurement, diluted with 0.9 % saline if necessary and measured in triplicates.

4.3. pH measurement

The pH values were determined with a Hanna pH meter (HI 2211 + HI1053). For this, 1 ml of the sample solution was placed in a test tube and the pH value was measured after a latency time of 10 min. On each measuring day, the pH meter was calibrated with ready-to-use buffer solutions (ROTI®Calipure).

4.4. HPLC

The HPLC system consisted of a degasser (Biotech Degasi® Classic), Waters™ 1525 binary HPLC pump, Waters™ 717plus autosampler, column oven (Techlab GmbH) and a Waters™ 2487 Dual λ absorbance detector (254 nm, 270 nm). An Agilent® Poroshell 120EC-C18, 2.7 µm, 3.0 mm × 50 mm column with Phenomenex® SecurityGuard™ Ultra cartridge, UHPLC C18 3.0 mm inclusive guard holder was used. The flow rate was set to 0.8 ml/min and a complex gradient was used. Mobile phase A consisted of a 10 mM sodiumphosphate buffer pH 3.2 and mobile phase B consisted of acetonitrile/sodiumphosphate buffer pH 3.2 60:40. The gradient started at 95 % A for 3 min, then changed to 80 % A within 2 min to continue to 40 % A until 16 min. Within another 2 min the mobile phase A decreased further to 30 % to remained there for 1 min. Then, the gradient changed within 1 minute to 95 % A and the re-equilibration started for 5 min. The entire gradient, including re-equilibration, ran for 25 minutes. The column oven was set to 20 °C. Waters™ Breeze™-software (version 3.3) was used for data acquisition and processing.
The HPLC method was validated according to ICH guideline. Therefore the linearity of the method was determined by a five-point calibration and the precision and reproducibility were checked measuring the maximum, middle and minimum concentrations of the range in triplicates. To validate the freezing process, the maximum and minimum concentrations were measured both before and after freezing and thawing in triplicates.

4.5. LC-MS coupling

LC-MS measurements were performed with an Agilent 1260 HPLC system (G1379B 1260 µ-degasser, G1312B bin pump, G1367E 1260 HiP ALS + G1330B, G1316A 1260 TCC, G4212B 1260 diode array detector) with gradient elution (A: 0.2 % acetic acid in water – B: 0.2 % acetic acid in acetonitrile) coupled to a Bruker Amazon SL mass spectrometer (Bruker Daltonik, Bremen, Germany) with an ion trap and MS^n facility. The gradient started with a flow rate of 1 ml/min at 95 % A for 2 min, then continued to 65 % A in 0.5 min and further to 20 % A in 3.5 min. The gradient changed to 3 % A in 0.5 min and remained there for 1.5 min. Finally, the gradient changed to 95 % A in 0.5 min and re-equilibration started at an increased flow rate of 1.3 ml/min for 1 min. The MS was switched on from min 2 to min 9.2 in positive mode. The column oven was set to 25 °C. A Phenomenex® Luna® 3 µm C18(2) 100 Å, 100 mm × 4.6 mm column inclusive Phenomenex® SecurityGuard™ Ultra cartridge, UHPLC C18 3.0 mm with guard holder was used. Hystar 3.2 SR 2 software was used to control the HPLC, Bruker Trap Control 7.0 software was used to control the MS and the MS data were analysed with Bruker Data Analysis 4.0.

4.6. Literature research

The chemical structure databases Reaxys® and Scifinder® were used to search for potential structures for the unknown compound.

4.7. Sample isolation for nuclear magnetic resonance spectroscopy

To isolate the relevant substance, the sample was obtained after several runs on an analytical HPLC column according to the procedure described above. The resulting sample solution was freeze-dried by the manufacturer's standard programme (Christ Alpha 2-4 LSCplus). After washing with D_2O and freeze-drying again, the sample was taken up in a mixture of D_2O and deuterated acetonitrile (1:1). This solution was used for all NMR experiments.

4.8. Nuclear magnetic resonance spectroscopy

NMR spectra were recorded using a Bruker Avance III 400 NMR spectrometer (Bruker, Rheinstetten, Germany) operating at 400.33 MHz for the proton channel and at 100.66 MHz for the ^{13}C channel by means of a 5 mm PABBO broad-band probe with a *z* gradient unit. Measurements were performed at 298 K. Automatic tuning and matching of the probe was performed, as was automatic shimming of the on-axis shims (*z* to z^5). The Bruker Topspin software 3.6.0 was employed for spectra recording. The manufacturer´s pulse programs were used. q^{1}H NMR using the ERETIC method allowed the estimation of the sample amount. The HH-COSY spectrum was recorded with presaturation of the remaining water signal, additionally $^1H^{13}C$ HSQC and ^1H ^{13}C HMBC spectra (optimized for 6 Hz and alternatively for 12.8 Hz CH long range couplings) were recorded for structure identification and the determination of ^{13}C chemical shifts. All spectra and ^1H and ^{13}C shift data were calibrated to an external solution of sodium 3-trimethylsilyl propane sulfonate in D_2O.

Data availability: The data of this study are available on request from the corresponding author.

Conflicts of interest: The authors declare that there is no conflict of interest regarding the publication of this article.

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