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Fmoc solid-phase synthesis of RF9 optimization with mass spectrometry verification

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ABSTRACT

The RF9 compound, which is an antagonist of the FF neuropeptide receptors is used as a therapeutic substance to improve the effectiveness of opioids in the chronic treatment of pain. The purpose of this study was to find the most efficient method of RF9 synthesis. The optimization experiment involved solid-phase peptide synthesis. The Fmoc strategy is based on the usage of the 9-fluorenylmethoxycarbonyl group to block reactive amino groups. Commonly applied RF9 synthesis is based on DIC/HOBt activation of 1-adamantanecarboxylic acid prior to its substitution. The experiments carried out in this research were based on the routinely applied DIC/HOBt carboxylic group activation and this scheme was compared with the COMU/DIPEA and DIC approach. The obtained results showed that COMU/DIPEA was the most efficient and effective method of RF9 synthesis. Using this strategy, pure compound was obtained, without any by-products, and at a highest yield. The use of COMU/DIPEA can be an excellent alternative to the routinely used RF9 synthesis.

INTRODUCTION

The safe use of opioid drugs is still one of the greater challenges in modern medicine. They are effective analgesics, widely used to relieve chronic and severe pains. However, these drugs raise some concern and controversy due to their possible side effects, such as tolerance, which accrues after repeated dosage. This phenomenon originates from the adaptive modifications in cellular responsiveness and, particularly, desensitization and down-regulation of opioid receptors, leading to tolerance [1-3]. Not only a decrease in cellular responsiveness, but also up-regulation of anti-opioid systems with pronociceptive properties may lead to the development of tolerance. Stimulation of opioid receptors initiates activation of anti-opioid systems that, in turn, induces long-lasting enhancement in pain sensitivity, and thus leading to hampered analgesic effect of the opioid receptor agonists [4]. Hence, it has been predicted that the expression of tolerance to analgesic effect of opioids, may be diminished by drugs that oppose opioid-induced hyperalgesia.

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Several neuromodulator systems, including NPFF (neuropeptide FF) system, have been shown to display anti-opioid properties [4,5]. Simonin *et al.* have noted, for example, that RF9 (1-Adamantanecarbonyl-RF-NH_2) exhibits antagonistic properties towards NPFF [4]. Reports also suggest that RF9 blocks the delayed and long-lasting heroin-induced hyperalgesia and associated tolerance. Similar results have been noticed in case of fentanyl or morphine treatment [6-9]. Moreover, pretreatment with RF9 prevents the antiopioid activities of the NPFF moiety and endogenous NPFF systems and might further potentiate the **μ-opioid antino**ciception of DN-9 (the multifunctional peptide analogue Tyr-D.Ala-Gly-NMe.Phe-Gly-Pro-Gln-Arg-Phe-NH_2) after icv administration [10]. Wang *et al*. in turn, contribute that the RF9 (the NPFF receptors antagonist) attenuates the hypothermia caused by morphine and nociceptin/orphanin FQ (two different opioid agonists). However, the hypothermia evoked by the central administration of NPFF was not affected by RF9 [11]. Based on the above observations, the efficient synthesis of this compound seems to be important for the more efficient therapeutic strategy using opioids.

In this paper, we present an optimization of the solid phase RF9 synthesis. As suggested in literature, the high

antagonistic potency of RF9 *in vivo* could be explained by its N-terminal modification, which ensures partial resistance against proteolytic enzymes [4]. Therefore, we were interested in investigating the efficacy of 1-adamantanecarboxylic acid substitutions in the RF9 structure. Reports on excellent coupling performance of a newer activation reagent COMU ((1-cyano-2-ethoxy-2-oxoethylidenaminooxy) dimethylamino-morpholino-carbenium hexafluorophosphate) and a less hazardous safety profile than benzotriazole-based reagents, have prompted us to investigate this activator [12]. In our work, we compared its efficacy with the routinely used DIC (N,N'-diisopropylcarbodiimide) and DIC/HOBt (N,N'-diisopropylcarbodiimide/N-hydroxybenzotriazole) carboxylic group activators [13,14]. To evaluate the coupling efficiency, we employed the commonly used quantitative Chloranil test [15] in combination with detection by mass spectrometry.

MATERIALS AND METHODS

Chemicals

The following chemicals were used during the experiments: 1-Cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium hexafluorophosphate was purchased from Merck (Germany), N-hydroxybenzotriazole (HOBt) was from Advanced ChemTech (USA), TentaGel S RAM-Phe Fmoc resin from Rapp Polymere (Germany), acetonitrile (ACN) and methanol from J.T. Baker (Poland), 2,3,5,6-tetrachlorocyclohexa-2,5-diene-1,4-dione (chloranil), acetaldehyde, N,N′-diisopropylcarbodiimide (DIC), N,N-dimethylphormamide (DMF), 1-adamantanecarboxylic acid and triisopropylsilane (TIS) from Fluka (Germany), dichloromethane (DCM), N,N-diisopropylethylamine (DIPEA), diethyl ether, phenol, Fmoc-Arg(Pbf)-OH, formic acid (FA), acetic acid, trifluoroacetic acid (TFA), and piperidine from Sigma-Aldrich (Germany).

Instrumentation

The mass spectrometry measurements were performed with the aid of two instruments. One was the Esquire 3000 from Bruker Daltonics, Bremen, Germany equipped with an electrospray (ESI) ion source. The second mass spectrometer applied in this research was MALDI TOF-TOF instrument ultrafleXtreme from Bruker Daltonics, Bremen Germany.

Fmoc solid-phase synthesis

The syntheses were carried out in polystyrene syringes with glass filter. Each of the three syntheses differed in the acylation method at the last stage of addition of 1adamantanecarboxylic acid. Three activating agents were used: COMU/DIPEA, DIC/HOBt and DIC, as described earlier.

The synthesis was carried out on TentaGel S RAM-Phe Fmoc resin as a solid support. The functional groups density of the resin was 0.26 mmol/g, while 0.3 g of the resin was used for each synthesis, thus the number of theoretical moles of each product was 0.078 mmol. The three steps in the synthesis of the RF9 compound are described below.

Acylation

The acylation reaction during RF9 synthesis was performed twice. Firstly, to introduce arginine: 151.8 mg of Fmoc-Arg(Pbf)-OH was mixed with threefold excess of COMU and sixfold excess of DIPEA. The reaction was performed for 2 hours at room temperature with slight mixing. Afterwards, the resin was washed three times with 2 mL of DMF for 2 minutes, 3 times with 2 mL of DCM for 2 minutes and finally, three times with DMF for 1 minute.

Secondly, an acylation reaction was performed to introduce 1-adamantanecarboxylic acid: for each of the three activating agents, 42.2 mg of 1-adamantanecarboxylic acid was used. A combination of three activating agents was tested:

- threefold excess of COMU and sixfold excess of DIPEA,
- threefold excess of DIC and threefold excess of HOBt,
- threefold excess of DIC.

Each reaction was performed in 2 mL DMF for 2 hours. Subsequently, the resin was washed in the same way, as described earlier after the first acylation reaction. Each acylation after washing was followed by a chloranil test.

Deprotection

Deprotection was performed twice by using 2 mL of 20% piperidine solution in DMF for 10 minutes. After deprotection, the resin was washed three times with 2 mL DMF for 2 minutes, 3 times with 2 mL DCM for 2 minutes and finally, three times with DMF for 1 minute. Afterwards, a chloranil test was performed.

Cleavage

Before cleavage, the resin was washed twice with 2 mL methanol for 10 minutes and dried in a SpeedVac for 18 hours. The cleavage cocktail consisted of a mixture of TFA/phenol/water/TIS at the ratio 88:5:5:2 (V/m/V/V). The cleavage was performed twice with 2 mL of the cleavage cocktail for 2 hours and 10 minutes, respectively. Note: TFA generates an acidic environment so as to enable the cleavage of the peptide from the resin with simultaneous removal of the Pbf protecting group. TFA also increases the solubility of peptides, while phenol, water and TIS act as free radical scavengers, thus preventing unwanted side-reactions that may occur during the cleavage step.

The excess of TFA was evaporated under a stream of nitrogen for about 10 minutes, to concentrate the product, which was precipitated by the addition of diethyl ether previously cooled to -20°C.

The samples were then stored under -20°C for 30 minutes to increase precipitation yield. After that, samples were centrifuged at 5000 RPM for 10 minutes at 4°C. The obtained pellets were dissolved in 80 µL of glacial acetic acid and lyophilized.

Chloranil test

The chloranil test was performed to establish whether amino groups ($-NH_2$) in the synthesized compound are free or blocked [15]. Briefly, a few grains of the resin were taken via syringe and placed in a glass test tube. Following this, 50 µl of a 2% solution of chloranil in DMF and 50 µl of 2% solution of acetaldehyde in DMF were added. The color

of the resin beads was controlled after 3 minutes. When the resin beads turn yellow, the -NH_2 groups are considered to have been blocked, which indicates that all amine groups had underwent substitution reactions. In contrast, a blue-green color means that still there are some -NH_2 groups present.

Mass spectrometry analysis

For the electrospray ionization mass spectrometry analyses, the products obtained by various methods were dissolved in 500 µL 50% ACN with 0.1% FA in water to obtain concentration equal to ca. 10 µg/mL. Before mass spectrometry analyses, the samples were placed in an ultrasonic bath for 15 minutes. The mass spectra were obtained in the positive ion mode. The instrument parameters were as published earlier [16]. Briefly, trap target was set to 30 000, max accumulation time: 200 ms, scan range: 100-1000 m/z, 10 mass spectra were averaged. The ion source parameters were as follows: capillary voltage: -4.5 kV, nebulizing gas pressure: 12 psi, drying gas flow rate: 8 L/min, drying temperature: 280°C. Air was used as a nebulizing gas [17]. All samples were introduced into the mass spectrometer at a flow rate of 3 µL/min.

For the MALDI TOF mass spectrometry, the samples were prepared using the standard dried droplet method, with α-cyano-4-hydroxycinnamic acid (CHCA) as a matrix. Mass spectra were acquired in the positive ion mode with an ion source voltage set to 25 kV. Fragmentation mass spectra were acquired by applying the post source decay (PSD).

RESULTS AND DISCUSSION

Synthesis of RF9 was performed in three syringes with application of a different activating agent during the last stage of the synthesis. Three activating agents were tested: (1-cyano-2-ethoxy-2-oxoethylidenaminooxy) dimethylamino-morpholino-carbenium hexafluorophosphate with N,N-diisopropylethylamine (COMU/DIPEA), N,N′-diisopropylcarbodiimide with N-hydroxybenzotriazole (DIC/HOBt) and N,N′-diisopropylcarbodiimide (DIC). For more details, see Materials and Methods.

Chloranil test

Figure 1 shows the result of the chloranil test after termination of the last stage of the synthesis (1-adamantanecarboxylic acid substitution). The test was negative after the application of COMU/DIPEA activation (yellow color), positive after the application of DIC (intense green color), and ambiguous after the application of DIC/HOBt (slightly green color, visible after several minutes). After the first synthesis step, the chloranil test for all samples was negative.

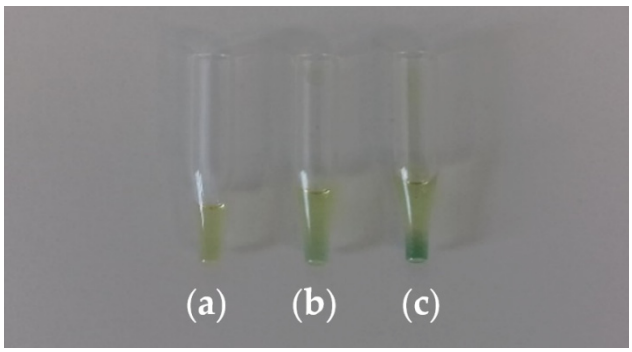

Figure 1. Chloranil test after 1-adamantanecarboxylic acid substitution with carboxylic acid activation by: (a) COMU/DIPEA, (b) DIC/HOBt and (c) only DIC

Mass spectra analysis

For the corresponding m/z values, the mass spectra measured for the products obtained with different methods of 1adamantanecarboxylic acid activation revealed a diverse range of m/z ratios and signal intensities (Fig. 2).

For COMU/DIPEA activating agent, the mass spectrum shows one peak at 483.3 m/z. This peak represents RF9 (Fig. 2(a)), and the spectrum confirms the presence of a pure compound obtained by this method.

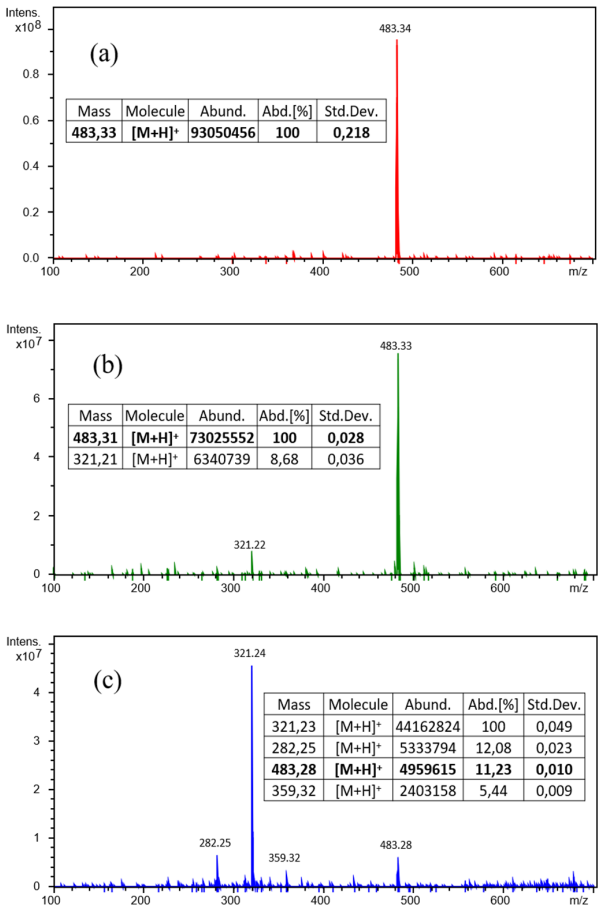

Figure 2. ESI mass spectra obtained for the RF9 synthesis after different carboxylic acid activation methods at the last stage of the synthesis: (a) COMU/DIPEA, (b) DIC/HOBt and (c) only DIC. Inserted tables contain information higher than 5% of relative abundance. The peak corresponding to a desired product is bolded in the tables

The mass spectrum of the sample after application of DIC/HOBt (Fig. 2(b)) shows two peaks, one at 483.3 m/z (ion of RF9) and the other at 321.3 m/z. This represents the presence of Arg-Phe-NH_2, a side compound obtained during the synthesis. This peak indicates that the addition of the 1-adamantanecarboxylic acid was not 100% efficient. Structures of these ions are presented in Figure 3.

Mass spectrum of the sample obtained when DIC was used shows more peaks (Fig. 2(c)). The peak at 483.3 m/z is

the RF9 compound ion. The peak at 321.3 m/z is the side product Arg-Phe-NH_2. Additionally, the peaks at 282.3 m/z and 359.3 m/z indicate the presence of impurities from the solvent used for synthesis.

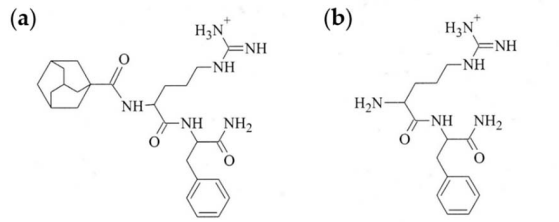

Figure 3. **(a)** Ion of the main product (RF9) detected in the mass spectra at m/z equal to 483.3 and **(b)** ion of the main impurity acquired in the mass spectra at m/z equal to 321.3 generated when the 1-adamantanecarboxylic acid was not completely substituted in the last step of the synthesis

In all 3 cases, the presence of RF9 was additionally verified by MS/MS experiments performed for the 483.3 m/z parent ion. Data were acquired using electrospray ionization (data not shown) and MALDI-TOF (Fig. 4).

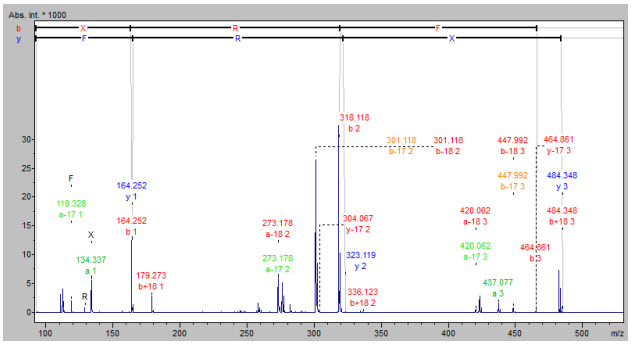

Figure 4. MS/MS MALDI-TOF mass spectrum of a protonated molecule of RF9 (m/z 483.3) confirming its presence after the synthesis. Data shown for COMU/DIPEA method. X on the mass spectrum stands for 1-adamantanecarboxylic acid residue

Yield of synthesis

The yields and masses of the obtained products for each synthesis method are presented in Table 1. However, the pure compound (RF9) was obtained without any side-products and impurities only in the case of activation with COMU/DIPEA. The synthesis yield for this reaction was highest and equals 37.2%. In case of short-sequenced peptides, precipitation may be difficult, and this may lead to decreased synthesis yield.

Table 1. Yield of the synthesis after application of different carboxylic acid activation agents

1-adamantane carboxylic acid activation method	Obtained product mass [mg]	Yield
COMU/DIPEA	15.76	37.2%
DIC/HOBt	11.19	26.4%
DIC	12.13	28.6%

The purity of the obtained compound can be estimated based on the mass spectra presented in Figure 2. The 100% abundance for peak 483.3 m/z that corresponds to the protonated RF9 molecule, was obtained only for the COMU/DIPEA and DIC/HOBt activation method of 1adamantanecarboxylic acid. However, the obtained product is pure only when the first method (COMU/DIPEA) was applied, as no other peaks with relative intensity higher than 5% are observed.

CONCLUSIONS

The aim of the study was to establish the most efficient method for RF9 synthesis. In particular, in the undertaken procedures, the activation of the 1-adamantanecarboxylic acid used at the last step of the solid phase synthesis was focused upon. RF9 is an antagonist of FF neuropeptide receptors, and therefore it may find application as an effective therapeutic agent for enhancing the efficacy of opioids in the chronic treatment of pain. RF9, by blocking the action of FF neuropeptide receptors, inhibits the development of tolerance to opioids.

The paper also describes the steps of peptide synthesis on a solid phase support. This method is now widely used because, unlike the classical synthesis in solution, it is faster, easier, and does not require purification after synthesis. Particular attention has been paid to the carboxyl activating agents used for the reaction.

The experimental part of the work presents three RF9 synthesis approaches. Each of these differed in the acylation method at the stage of 1-adamantanecarboxylic acid substitution. For this purpose, activators of the carboxyl group, such as DIC/HOBt (commonly used), were compared with COMU/DIPEA. At each step of the synthesis, the chloranil test was performed, which allowed assessing whether the amino groups were free or blocked to assign reaction yield.

The final products of all syntheses were analyzed by mass spectrometry with electrospray ionization (ESI) and MALDI-TOF. After analysis, it has been clearly concluded that the COMU/DIPEA activation method was the most effective. Pure RF9 was obtained only with this method, with no side-products or impurities. In contrast, while the standard DIC/HOBt method resulted in synthesis of the main compound, it also resulted in the generation of the side product Arg-Phe-NH_2 (with no 1-adamantanecarboxylic acid attached). The use of DIC alone gave the worst result. Herein, besides the signal from the main compound, peaks indicating the presence of impurities in the mass spectrum were observed. The highest yield of synthesis equal to 37.2% was obtained for COMU/DIPEA method.

Summarizing, the obtained results allowed the finding of a more efficient method of RF9 synthesis than that routinely used. COMU is a more effective carboxyl activator than DIC when considering 1-adamantanecarboxylic acid activation. In addition, the use of the racemization blocking agent HOBt, which minimizes the risk of generation of other optical isomers, is not required.

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CONFLICTS OF INTEREST

The authors do not declare any conflict of interests that could affect the objectivity and credibility of the work.

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REFERNCES

1. Wojciechowski P, Andrzejewski K, Kaczyńska K. Intracerebroventricular neuropeptide FF diminishes the number of apneas and cardiovascular effects produced by opioid receptors' activation. *Int J Mol Sci*. 2020;21:8931.
2. Elhabazi K, Trigo JM, Mollereau C, Moulédous L, Zajac JM, Bihel F, et al. Involvement of neuropeptide FF receptors in neuroadaptive responses to acute and chronic opiate treatments. *Br J Pharmacol*. 2012;165(2):424-35.
3. Hartman K, Mielczarek P, Smoluch M, Silberring J. Inhibitors of neuropeptide peptidases engaged in pain and drug dependence. *Neuropharmacology*. 2020;175:108137.
4. Simonin F, Schmitt M, Laulin JP, Laboureyras E, Jhamandas JH, MacTavish D, et al. RF9, a potent and selective neuropeptide FF receptor antagonist, prevents opioid-induced tolerance associated with hyperalgesia. *Proc Natl Acad Sci USA*. 2006;103(2):466-71.
5. Grasso G, Mielczarek P, Niedziolka M, Silberring J. Metabolism of cryptic peptides derived from neuropeptide FF precursors: the involvement of insulin-degrading enzyme. *Int J Mol Sci*. 2014;15(9):16787-99.
6. Fang Q, Wang YQ, He F, Guo J, Guo J, Chen Q, Wang R. Inhibition of neuropeptide FF (NPFF)-induced hypothermia and anti-morphine analgesia by RF9, a new selective NPFF receptors antagonist. *Regul Pept*. 2008;147(1-3):45-51.
7. Zhang T, Han Z, Shi X, Zhao W, Wang Z, Zhang R, et al. Discovery of two novel branched peptidomimetics containing endomorphin-2 and RF9 pharmacophores: Synthesis and neuropharmacological evaluation. *Bioorg Med Chem*. 2019;27(4):630-43.
8. Hammoud H, Elhabazi K, Quillet R, Bertin I, Utard V, Laboureyras E, et al. Aminoguanidine ydrazone derhivatives as nonpeptide NPFF1 receptor antagonists reverse opioid induced hyperalgesia. *ACS Chem Neurosci*. 2018;9(11):2599-609.
9. Kotlinska JH, Gibula-Bruzda E, Koltunowska D, Raoof H, Suder P, Silberring J. Modulation of neuropeptide FF (NPFF) receptors influences the expression of amphetamine-induced conditioned place preference and amphetamine withdrawal anxiety-like behavior in rats. *Peptides*. 2012, 33(1):156-63.
10. Wang ZL, Pan JX, Song JJ, Tang HH, Yu HP, Li XH, et al. Structure-based optimization of multifunctional agonists for opioid and neuropeptide FF receptors with potent nontolerance forming analgesic activities. *J Med Chem*. 2016;59(22):10198-208.
11. Wang YQ, Guo J, Wang SB, Fang Q, He F, Wang R. Neuropeptide FF receptors antagonist, RF9, attenuates opioid-evoked hypothermia in mice. *Peptides*. 2008;29(7):1183-90.
12. El-Faham A, Albericio F. COMU: a third generation of uronium-type coupling reagents. *J Pept Sci*. 2010;16(1):6-9.
13. El-Faham A, Subirós-Funosas RS, Prohens R, Albericio F. COMU: A safer and more effective replacement for benzotriazole-based uranium coupling reagents. Chem A Eur J. 2009;15:9404-16.
14. Hartman K, Mielczarek P, Silberring J. Synthesis of the novel covalent cysteine proteases inhibitor with iodoacetic functional group. *Molecules*. 2020;25(4):813.
15. Christensen T. Qualitative test for monitoring coupling completeness in solid phase peptide synthesis using chloranil. *Acta Chem Scand B*. 1979;33:763-6.
16. Mielczarek P, Raoof H, Kotlinska JH, Stefanowicz P, Szewczuk Z, Suder P, et al. Electrochemical simulation of cocaine metabolism-a step toward predictive toxicology for drugs of abuse. *Eur J Mass Spectrom* (*Chichester*). 2014;20(4):279-85.
17. Mielczarek P, Silberring J, Smoluch M. Technical note: Air compared to nitrogen as nebulizing and drying gases for electrospray ionization mass spectrometry. *Eur J Mass Spectrom* (Chichester). 2016;22(5):213-6.