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Application of LC-NMR and HR-NMR to the characterization of biphenyl impurities in the synthetic route development for vestipitant, a novel NK1 antagonist

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ABSTRACT

Vestipitant (**1**) is a novel NK1 antagonist currently under investigation for the treatment of CNS disorders and emesis. The first synthetic step comprised a Grignard synthesis. An impurity was identified and initially expected to be a symmetric biphenyl. This paper reports the work to synthesise the supposed structure and the spectroscopic analyses (LC-NMR and HR-NMR) to correctly identify the real structure and understand the chemical pathway of the impurity.

1. Introduction

Substance P (SP) is a member of the neurokinin family that exerts its pleiotropic role by preferentially binding to the neurokinin receptor classified as NK1. The role of SP in mood disorders may also partly depend on its interaction with serotonin (5-HT) receptors. Preclinical studies suggest that NK1 antagonists and selective serotonin reuptake inhibitors (SSRIs) may synergise to produce a final common effect on serotonergic neurotransmission. Furthermore, SP and the NK1 receptors that mediate its activity are present in the brain stem centres that elicit the emetic reflex. Nausea and vomiting have been reported as the most distressing side effects of chemotherapy, and the disruptive effects of these symptoms on patients' daily lives have been well documented. In light of the need for continued routine use of emetogenic chemotherapy, effective prevention of chemotherapy-induced nausea and vomiting (CINV) is a central goal for physicians administering cancer chemotherapy. Vestipitant (**1**) is a novel NK1 antagonist currently under development both to tackle central nervous system (CNS) diseases and to relieve CINV symptoms [1].

The initial route that was employed to synthesize vestipitant (**1**) in grams scale is showed in Scheme 1. The present work describes the identification of a potentially mutagenic impurity which formed during the Grignard step and was observed in the isolated intermediate (**2**). Liquid chromatography NMR (LC-NMR) [2–4] and high resolution NMR (HR-NMR) were applied on both synthesized and purified standards. It is worth noting that in this case the transparency of the impurity to the mass detector did not allow to follow the strategy based on the combination of LC–MS and LC-NMR, frequently successful for impurities identification [5–7].

2. Experimental

2.1. LC-NMR

LC-NMR was performed on a Varian INOVA 600 MHz NMR spectrometer operating at 599.71 MHz for ^{1}H coupled to a Varian Pro-Star 5 HPLC system equipped with diode array detector (DAD). A PFG-IFC triple resonance (^{1}H, ^{13}C, ^{15}N) NMR probe was used. LC-NMR was performed in the stopped-flow and time-slice modes. The HPLC method was initiated with 70% D_2O containing 0.1% TFA–30% acetonitrile (ACN) containing 0.1% trifluoroacetic acid (TFA), followed by a gradient to 100% ACN containing 0.1% TFA over 9 min (total run time 15 min), with a flow rate of 1 ml/min and UV detection at 254 nm. The mother liquor sample (100 μl)

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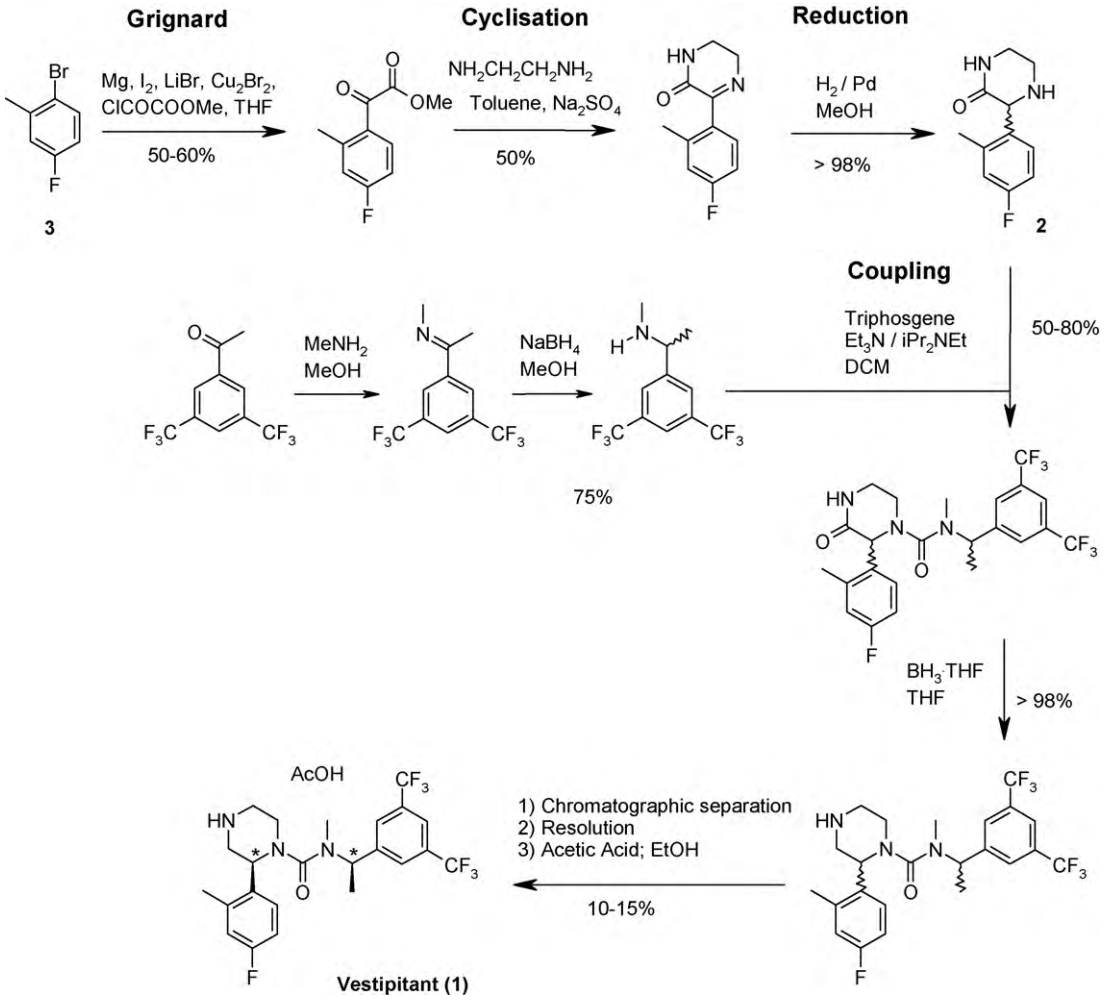
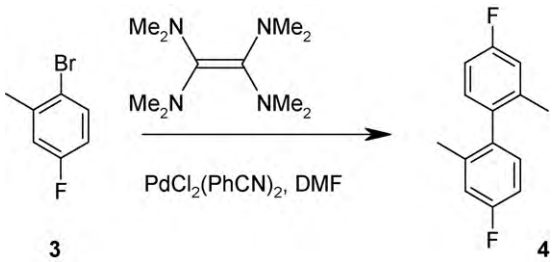


Scheme 1. Vestipitant (**1**) synthetic route.

was injected on to a reversed-phase column Varian Polaris A-C18 (150 mm × 4.6 mm × 5 μm). Solid samples were dissolved using the mobile phase in an approximate concentration of 1 mg/ml; injection volume and chromatographic conditions being the same as previously described. ^{1}H NMR spectra were collected on the impurities of interest (R_t = 9.5 min) by stopping the chromatographic flow in correspondence of the UV detection. During the time-slice experiment three ^{1}H NMR spectra were collected stopping the flow three times at an interval of 10 s starting from the initial UV detected elution of the chromatographic peak. ^{1}H NMR spectra were acquired using the standard WET1D sequence for achieving double pre-saturation of both residual water and ACN resonances. Data were acquired with 9–12 kHz sweep width using 32k time-domain points with an acquisition time of 1.82 s. Variable number of scans [64 for time-slice and stopped-flow LC-NMR with intermediate (**2**) mother liquor; 1024 for stopped-flow LC-NMR with isolated solid intermediate (**2**); 64 for stopped-flow LC-NMR with synthesized biphenyl (**4**)] were used upon the relative concentration of the impurities within the probe flow cell in order to achieve a sufficient signal-to-noise. All spectra were collected at operating temperature of 23 °C. ^{1}H LC-NMR spectra were referenced to the ACN resonance (1.96 ppm).

2.2. HR-NMR

Samples of the compounds obtained from synthesis (**4**) or from chromatography purification (**6**, **7**) were dissolved in either DMSO-d_6 or CD_3CN. 1D ^{1}H, ^{13}C and ^{19}F NMR, as well as 1D NOESY, 2D ^{1}H–^{13}C gHSQC, 2D ^{1}H–^{13}C CIGAR-HMBC, 2D ^{1}H–^{13}C gHSQC-TOCSY [8] spectra were acquired on a Varian INOVA 600 MHz NMR spectrometer operating at 599.71 MHz for ^{1}H, 150.81 MHz for ^{13}C and 564.22 MHz for ^{19}F, respectively. A PFG 5 mm triple resonance cold probe was used and all spectra were collected at operating temperature of 25 °C. ^{1}H NMR spectra were acquired with a 9 kHz sweep width using 32k time-domain points with an acquisition time of 3.56 s. ^{13}C NMR spectra were acquired with a 28 kHz sweep width using 32k time-domain points with an acquisition time of 1.37 s. ^{19}F NMR spectra were acquired with a 30 kHz sweep width using 32K time-domain points with an acquisition time of 0.64 s. ^{1}H and ^{13}C NMR spectra were referenced to residual solvent lines (2.50/39.51 ppm for DMSO-d_6 and 1.96/1.39 ppm for CD_3CN,



Scheme 2. Synthesis of the symmetric biphenyl (**4**).

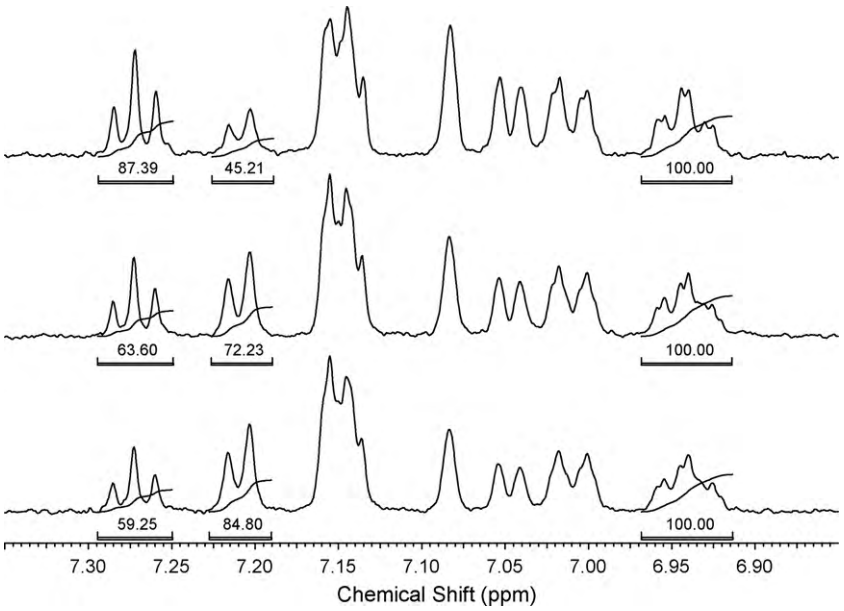


Fig. 1. Aromatic region expanded plot of the time-slice LC-NMR spectra the intermediate (**2**) mother liquor impurity fraction. The three spectra were collected stopping the flow at an interval of 10 s starting from the initial impurity peak elution (top). The different integration of the two isolated downfield resonances demonstrates that they belong to two different co-eluting species.

respectively). ^{19}F NMR spectra were referenced to external trifluoroacetic acid (−76.5 ppm). NOESY1D experiments were collected by selective irradiation of some resonances using the standard sequence available in the Varian vnmr6.1 package and processed applying a weighting function *lb* = 1. The 2D ^{1}H–^{13}C gHSQC spectra were acquired using ^{1}H sweep width 5 kHz, ^{13}C sweep width 24 kHz, with 1024 points in *f*2, 256 complex increments in *f*1, two scans per increment, spectral editing (CH_2 negative, CH/CH_3 positive). The 2D ^{1}H–^{13}C CIGAR-HMBC spectra were acquired using ^{1}H sweep width 5 kHz, ^{13}C sweep width 29 kHz, with 1024 points in *f*2, 256 complex increments in *f*1, 8 scans per increment, optimization for ^{n}J(C,H) of 8 Hz. The 2D ^{1}H–^{13}C gHSQC-TOCSY spectrum was acquired using ^{1}H sweep width 5 kHz, ^{13}C sweep width 24 kHz, with 1024 points in *f*2, 512 complex increments in *f*1, eight scans per increment, optimization for ^{1}J(C,H) of 150 Hz, spin-lock mixing time of 150 ms at a strength of 8 kHz with a MLEV-17

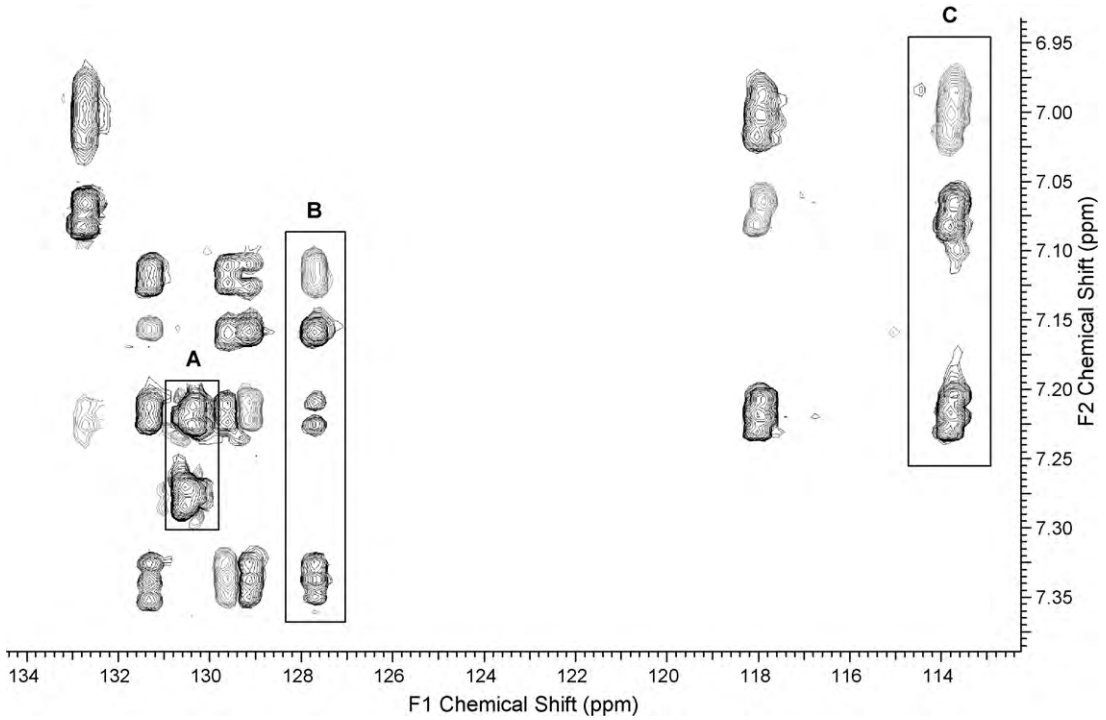


Fig. 2. Aromatic region expanded plot of the ^{1}H–^{13}C gHSQC-TOCSY experiment performed on the purified impurity fraction. The light grey spots represent ^{1}H–^{13}C one bond couplings, the dark grey spots represent the TOCSY correlations in ^{1}H and ^{13}C dimensions, respectively. The boxes A, B and C highlight the spin systems which were identified. The ^{1}H NMR integration (see also Fig. 1) allowed understanding that the box C highlighted resonances were constituted by the overlapping of one proton for each species. The box A highlights two cross-peaks only, which show the typical shape of protons belonging to *para*-disubstituted aryl systems.

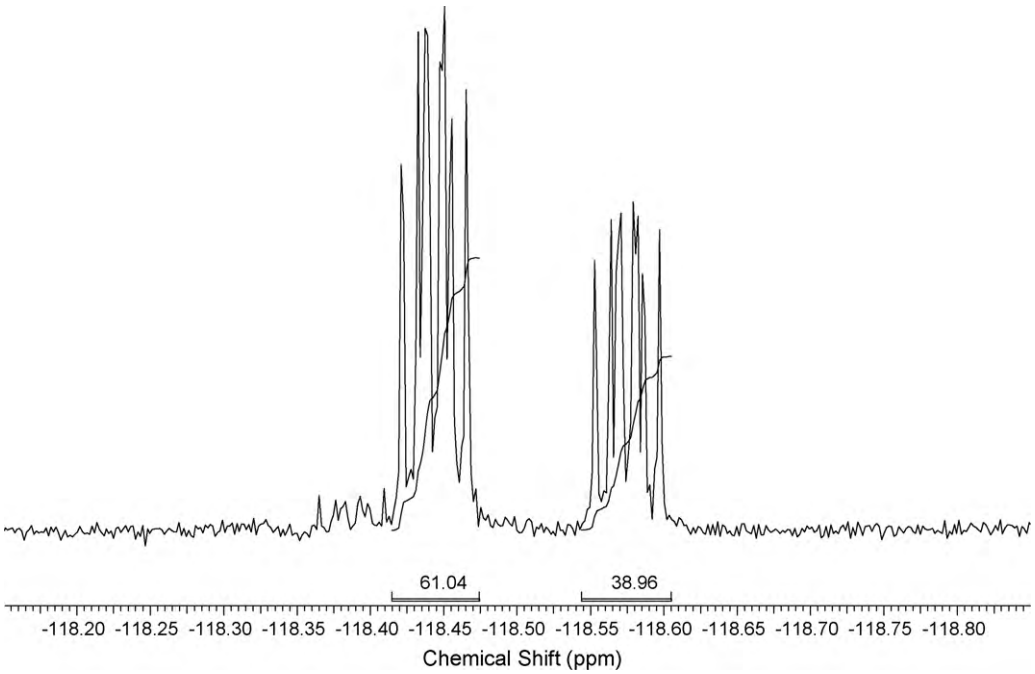


Fig. 3. ^{19}F NMR spectrum collected on the purified impurity fraction. The two compounds in mixture (*ca.* 60/40 molar) are both bearing fluorine in a very similar structural context (same $^{1}H–^{19}F$ coupling constants).

modulation, spectral editing (TOCSY negative, HSQC positive). The phase-sensitive spectra ($^{1}H–^{13}C$ gHSQC and $^{1}H–^{13}C$ gHSQC-TOCSY) were transformed with a cosine squared weighting function in both dimension, after applying zero-filling in *f*2 and either zero-filling or linear prediction in *f*1. The magnitude mode experiments ($^{1}H–^{13}C$ CIGAR-HMBC) were transformed with a sine-bell weighting function in both dimensions after applying zero-filling in *f*1.

2.3. HPLC

The qualitative and quantitative HPLC analysis of the regioisomers of bromo-fluoro-toluene was performed on an Agilent system 1100 LC (Waldbronn, Germany) using a reversed-phase column Phenomenex Luna C18 (150 mm × 4.6 mm × 3 μm). The mobile phase consisted of two solutions, A: water + 0.05% TFA and B: ACN + 0.05% TFA. The method started with 100% A, followed by a gradient to 95% B over 30 min. The HPLC system operated at a flow rate of 1.0 ml/min, the detection wavelength was set at 210 nm and the temperature at 40 °C. Samples were prepared by dissolving about 5 mg of analyte in a 10 ml volumetric flask, adding 10 ml of a mixture 50/50% (v/v) of ACN and water then sonicated to get complete dissolution.

Typical retention times are the following: 1-bromo-4-fluoro- 2-methylbenzene (**3**), 24.32 min; 1-bromo-2-fluoro-4-methylbenzene (**8**), 24.02 min; 2-bromo-4-fluoro-1-methylbenzene, 24.58 min; 4-bromo-2-fluoro-1-methylbenzene, 24.68 min.

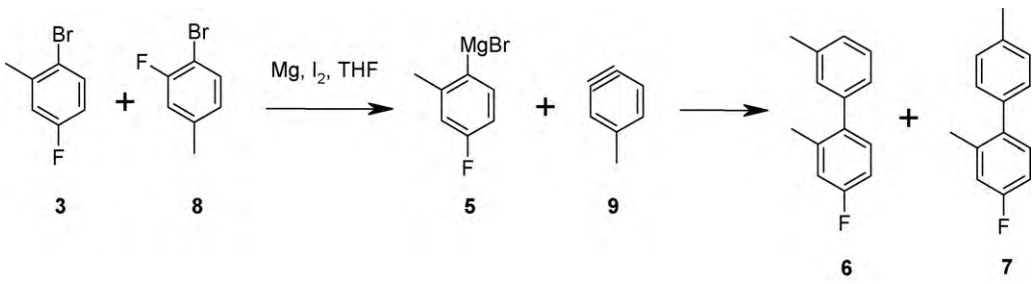
2.4. LC–MS

All MS data were collected in positive ion mode, with the following optimized ESI-MS parameter settings: capillary voltage: 3.5 kV; cone voltage: 30 V; extractor voltage: 2 V; desolvation gas flow (ultra pure nitrogen): 450 l/h; source temperature: 80 °C; desolvation temperature: 450 °C. Data were acquired by full scan monitoring in the range 100–1000 *m*/*z*. Data were acquired and processed using Empower 2 software (Waters, Milford, MA).

2.5. Materials

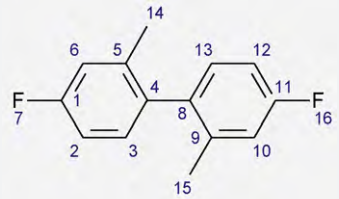
The symmetric compound 4,4′-difluoro-2,2′-dimethylbiphenyl (**4**) was prepared as follows: a mixture of 2-bromo-5-fluoro toluene (**3**) (1.0 g, 5.29 mmol), tetrakis(dimethylamino)ethylene TDAE (2.5 ml, 10.58 mmol), and $PdCl_2(PhCN)_2$ (0.1 g, 0.26 mmol) in DMF (5.0 ml) was heated at 70 °C overnight. Purification on silica pad using cyclohexane as eluant afforded biphenyl (**4**) in 50% yield [9].

The mixture of 4-fluoro-2,3′-dimethylbiphenyl (**6**) and 4-fluoro-2,4′-dimethylbiphenyl (**7**) was obtained by chromatography separation from intermediate (**2**) mother liquor.

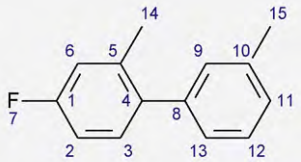


Scheme 3. Structure of the identified biphenyl structures (**6**) and (**7**) and the suggested benzyne mechanism which leads to their formation.

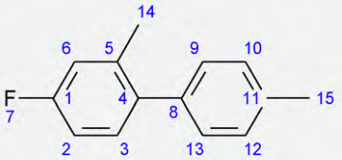
Table 1
NMR assignments for biphenyls (**4**), (**6**) and (**7**).



(4)



(6)



(7)

	^{1}H NMR (δ, ppm)[a]	J_{HH} (Hz)	J_{HF} (Hz)	^{13}C NMR (δ, ppm)[a]	J_{CF} (Hz)	^{19}F NMR (δ, ppm)[a]	^{1}H NMR (δ, ppm)[b]	J_{HH} (Hz)	J_{HF} (Hz)[c]	^{13}C NMR (δ, ppm)[b]	J_{CF} (Hz)	^{19}F NMR (δ, ppm)[b]	^{1}H NMR (δ, ppm)[b]	J_{HH} (Hz)	J_{HF} (Hz)[c]	^{13}C NMR (δ, ppm)[b]	J_{CF} (Hz)	^{19}F NMR (δ, ppm)[b]
1				161.32 (d)	242.7					162.90 (d)	243.5					162.86 (d)	242.9	
2	7.06 (td)	8.3, 2.3	8.3	112.43 (d)	20.8		7.00 (m)		8.4	113.37 (d)	21.4		7.00 (m)		8.4	113.38 (d)	20.8	
3	7.09 (dd)	8.3	6.3	131.99 (d)	8.7		7.21 (m)		6.5	132.30 (d)	8.5		7.21 (m)		6.5	132.36 (d)	8.6	
4				136.19 (d)	3.0					139.30 (d)	2.5					139.12 (m)	n.d.	
5				138.29 (d)	8.2					139.08 (m)	n.d.					139.05 (m)	n.d.	
6	7.17 (dd)	2.3	9.9	116.29 (d)	21.2		7.07 (m)		10.2	117.57 (d)	20.8		7.07 (m)		10.2	117.57 (d)	20.8	
7						−116.2 (m)						−118.4 (m)						−118.6 (m)
8				136.19 (d)	3.0					141.84 (s)						138.92 (s)		
9				138.29 (d)	8.2		7.15 (bs)			130.89 (s)			7.21 (m)			130.13 (s)		
10	7.17 (dd)	2.3	9.9	116.29 (d)	21.2					139.04 (s)			7.27 (d)	7.7		129.92 (s)		
11				161.32 (d)	242.7		7.21 (m)			128.75 (s)						129.92 (s)		
12	7.06 (td)	8.3, 2.3	8.3	112.43 (d)	20.8		7.33 (t)	7.4		129.20 (s)			7.27 (d)	7.7		129.92 (s)		
13	7.09 (dd)	8.3	6.3	131.99 (d)	8.7		7.11 (d)	7.4		127.27 (s)			7.21 (m)			130.13 (s)		
14	1.98 (s)			19.49 (d)	1.3		2.25 (s)			20.81 (d)	1.2		2.25 (s)			20.85 (d)	1.2	
15	1.98 (s)			19.49 (d)	1.3		2.397 (s)			21.51 (s)			2.401 (s)			21.22 (s)		
16						−116.2 (m)												

[a] Spectra collected in DMSO-d_6.

[b] Spectra collected in CD_3CN.

[c] Measured from ^{19}F NMR spectrum as ^{1}H NMR results in complex overlapping of the two regioisomers (**6**) and (**7**) in mixture. ^{13}C NMR resonances in the region 139.2–138.9 for regioisomers (**6**) and (**7**) in mixture are assigned by means of ^{1}H–^{13}C 2D NMR experiments; coupling constants in this region cannot be determined precisely due to complex overlapping (n.d.).

1-Bromo-4-fluoro-2-methylbenzene (**3**) (CAS 452-63-1), 1-bromo-2-fluoro-4-methylbenzene (**8**) (CAS 452-74-4), 2-bromo-4-fluoro-1-methylbenzene (CAS 1422-53-3), 4-bromo-2-fluoro-1-methylbenzene (CAS 51436-99-8) were all purchased by Aldrich and used without further purification.

3. Results and discussion

An unknown impurity was detected at the analytical controls applied on the isolated intermediate (**2**). This impurity, due to its retention time in the HPLC chromatogram and to its non-ionization in LC–MS, was suspected to be related to a biphenyl side-product deriving from the starting material (**3**). Some biphenyls are supposed to be mutagenic and are known to be persistent and bioaccumulative environmental toxicants [10]. As a consequence, deep investigation on the unknown impurity was undertaken. The initial Grignard step in the synthetic process illustrated in Scheme 1 could in theory lead to a symmetric biphenyl (**4**), which was initially considered as the most probable. The synthesis of the symmetric biphenyl (**4**) was then performed in order to verify the initial hypothesis (Scheme 2).

The compound (**4**) was characterized by HR-NMR and used as standard for co-injection during LC-NMR experiments as well.

The HPLC chromatogram of intermediate (**2**) and that of its mother liquor demonstrated that the impurity was not correspondent to the symmetric biphenyl (**4**), due to a slight difference in retention time [9.5 min for the impurity detected in intermediate (**2**) and 9.4 min for the standard of (**4**) using the LC-NMR experimental conditions, respectively]. This result was also confirmed by ^{1}H NMR spectra collected in stopped-flow LC-NMR mode after injection of the synthesized biphenyl (**4**), the intermediate (**2**), and its mother liquor. This result excluded then the risk of toxicity associated to the symmetric biphenyl (**4**), but forced further investigation on the nature of the unknown impurity in order to elucidate its structure, evaluate the potential associated risks, and in case set specific controls.

The stopped-flow LC-NMR experiments did not allow the immediate identification of the impurity due to complex aromatic region. However, two singlets (at 2.32 and 2.18 ppm, respectively) were detected in the aliphatic region consistent with aryl-CH_3 groups. These two signals did not showed a 1:1 integration (measured ratio approximately 40/60) leading to the hypothesis that we were in presence of a mixture of two closely related structures co-eluting in the HPLC run. The nature of the impurities could still be due to biphenyl structures as the observed resonances had no relationship with the structures of either the intermediate (**2**) or its non-isolated precursor (Scheme 1).

A clearer demonstration of this hypothesis was achieved by performing a time-slice LC-NMR experiment [11]. The flow of the chromatographic run was here stopped at regular intervals (10 s) during the elution of the impurity peak. The analysis of the aromatic region integrals in the resulting spectra demonstrated the co-elution of two compounds (Fig. 1).

The structural elucidation required a purification of the chromatographic peak, due to the complexity and to the low amount available in the NMR probe flow cell for performing 2D NMR experiments indispensable for getting structural data. After chromatography purification, an approximately 90% pure sample containing the two unknown impurities was analysed by means of HR-NMR experiments. Deuterated acetonitrile (CD_3CN) was chosen as solvent for NMR experiments with the purpose to get resonances as closest as possible to those observed in the LC-NMR conditions (the peak eluted in the LC-NMR run when the eluent composition was 100% ACN + 0.1% TFA). Despite a chemical shift difference was observed between the spectra collected in the two diverse conditions, due to both huge concentration difference and to the presence of residual water and TFA in the LC-NMR, the trend and multiplicity of the aromatic resonances remained the same. Moreover, two extra methyl lines for each component of the mixture were revealed in the HR-NMR spectra, being two of them not detected in the previous LC-NMR experiments as a consequence of their closeness to the suppressed ACN signal.

Both 1D (^{1}H, ^{19}F, ^{13}C NMR, NOESY1D) and 2D (gHSQC, CIGAR-HMBC, gHSQC-TOCSY) experiments were performed in order to get a full insight on the two unknown structures. More specifically, the ^{1}H–^{13}C gHSQC-TOCSY experiment resulted particularly helpful for providing information on both ^{1}H and ^{13}C NMR chemical shifts of the aromatic spin systems of the two compounds in mixture, allowing their identification (Fig. 2).

In addition, ^{19}F NMR resulted as a further proof that both compounds had single fluorine (Fig. 3) and that this fluorine is placed in a very similar aromatic system, as a result from the evaluation of measurable ^{1}H–^{19}F coupling constants.

The structures of the two co-eluting impurities were then suggested as two asymmetric biphenyls, reported in Scheme 3. Their formation is also suggested here, with the hypothesis that a regioisomer of the starting material 1-bromo-4-fluoro-2-methylbenzene (**3**) could be present as its contaminant: while the regioisomer (**3**) forms the expected Grignard reagent (**5**), the potential regioisomer, 1-bromo-2-fluoro-4-methylbenzene (**8**) eliminates MgBrF leading to the formation of the benzyne (**9**) [12]. Benzyne (**9**) is then able to react both in positions *meta* and *para* with respect to the methyl group, leading to the formation of biphenyls (**6**) and (**7**).

The commercially available 1-bromo-2-fluoro-4-methylbenzene (**8**) and other bromo-fluoro-methylbenzene regioisomers were then used to develop a HPLC method which enabled the demonstration that the regioisomer (**8**) was in fact a low-level impurity in the starting material (**3**).

The full ^{1}H, ^{13}C and ^{19}F NMR assignments for biphenyls (**4**), (**6**) and (**7**) are finally reported in Table 1.

Following the identification of the two biphenyls (**6**) and (**7**), these structures were submitted to the *in silico* DEREK for Windows program [13] in order to predict whether there could be a specific need for their quantification in the ppm level range with specific methods and to correctly address their analytical control in the process. DEREK for Windows is a rule-based expert system that predicts the toxicological hazard of chemicals based on an analysis of their molecular structure. The software uses structure activity relationships (SAR or structural alerts) and gives some consideration to physicochemical properties to derive its predictions. All outcomes are peer reviewed by expert toxicologists and are supported by literature references. Alerts cover a wide range of toxicological end points, including carcinogenicity, mutagenicity, and skin sensitisation. All the tools are provided for users to add their own in-house alerts.

The biphenyl structures resulted negative to this test, confirming that no direct activity is expected on the DNA chain.

4. Conclusions

The combination of LC-NMR and HR-NMR allowed addressing the potential flag of mutagenic impurities which form in vestipitant (**1**) early stage synthesis. The peculiar complexity due to co-elution in the available HPLC method as well as the transparency to MS detector was approached by means of an investigation using different LC-NMR methods. Only the further purification by chromatography allowed a full characterization of the two biphenyl compounds. The root cause of their presence was then demonstrated being due to a contamination of the starting material 1-bromo-4-fluoro-2-methylbenzene (**3**) with the regioisomer 1-

bromo-2-fluoro-4-methylbenzene (**8**). The completion of the identification activities allowed finally a correct design of the process and of the analytical control of these impurities, setting suitable limits for the wrong regioisomer in the starting material (**3**).

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