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Structural characterization of *N*-acyl-homoserine lactones from bacterial quorum sensing using LC-MS/MS analyses after Paternò-Büchi derivatization in solution

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Abstract: The bacterial quorum sensing is a chemical language allowing bacteria to interact through the excretion of molecules called auto-inducers, like *N*-acyl-homoserine lactones (AHLs) produced by Gram-negative *Burkholderia* and *Paraburkholderia* bacteria known as opportunistic pathogens. The AHLs differ in their acyl-chain length and may be modified by a 3-oxo or 3-hydroxy substituent, or C=C double bonds at different positions. As the bacterial signal specificity depends on all of these chemical features, their structural characterization is essential to have a better understanding of the population regulation and virulence phenomenon. This study aimed at enabling the localization of the C=C double bond on such specialized metabolites while using significantly low amounts of biological material. The approach is based on LC-MS/MS analyses of bacterial extracts after in-solution derivatization by a photochemical Paternò-Büchi reaction, leading to the formation of an oxetane ring and subsequently to specific fragmentations when performing MS/MS experiments. The in-solution derivatization of AHLs was optimized on several standards, and then the matrix effect of bacterial extracts on the derivatization was assessed. As a proof of concept, the optimized conditions were applied to a bacterial extract enabling the localization of C=C bonds on unsaturated AHLs.

Keywords: structural characterization; *N*-acyl-homoserine lactones; tandem mass spectrometry; Paternò-Büchi derivatization; liquid chromatography; double bond localization

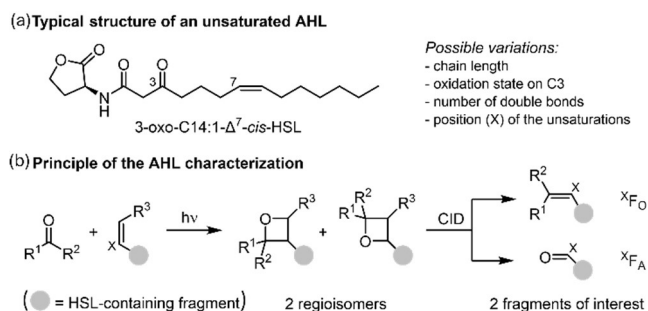
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44 1. Introduction

45 Quorum sensing is a widespread and well-known cell density-dependent mechanism, which allows
 46 prokaryotes to synchronize their metabolic and physiological activities at high cell concentrations [1].
 47 Quorum sensing system relies on the “sensing” of molecular indicators of bacterial densities. At a low
 48 cell density, these compounds, named autoinducers (AIs), are released in small amounts in the nearby
 49 environment of bacterial cells [2]. The concentration of AIs in the environment increases
 50 concomitantly with the cell concentration and induces intracellular signaling cascades in targeted cells
 51 at a certain threshold of detection [3]. These mechanisms lead to deep modifications of gene
 52 expression and large phenotypic transformations [4]. For some particular opportunistic human
 53 pathogens, these AI molecules regulate the expression of various virulence genes [5] and are involved
 54 in the bacterial antibiotic resistance and biofilm formation [6]. The characterization of AIs is therefore
 55 critical in a particularly alarming context of infectious diseases surge.

56 Species of the *Burkholderia* and *Paraburkholderia* genera are Gram-negative bacteria found in diverse
 57 environment niches. They are mainly non-pathogenic and plant-beneficial-environment species [7–9].
 58 However, some of these bacteria have been described as opportunistic pathogens for humans [10],
 59 including the *Burkholderia cepacia* complex that can cause life-threatening infections in
 60 immunodeficient patients [11]. The quorum sensing mechanism in *Burkholderia* and *Paraburkholderia*
 61 species, as in many Gram-negative bacteria [12], is based on *N*-acyl homoserine lactones (AHLs) [6].
 62 These well-described hydrophobic AIs are composed of a lactone ring linked to a fatty acid residue,
 63 with variations in acyl-chain length (generally from C4 to C18) and different oxygenated functions like
 64 3-oxo or 3-hydroxy substituents, as well as C=C double bonds at various positions on the carbon
 65 backbone chain (Fig. 1a) [13–15]. Fine structural characterization of AHLs is crucial for understanding
 66 population regulation and virulence as signal specificity depends on all of these features [16]. Indeed,
 67 the position of the double bonds, and all the other structural differences, could have an impact on the
 68 bacterial signal, since AHLs are detected by specific receptors. While tandem mass spectrometry
 69 (MS/MS) readily provides information on acyl-chain length and degrees of unsaturation [17],
 70 pinpointing C=C double bonds remains challenging due to limitations in classical fragmentation
 71 methods like Collision Induced Dissociation (CID) [18].

72 Alternative fragmentation methods have been developed to localize C=C bonds on lipid acyl chains,
 73 mostly on phospholipids, such as Ozone Induced Dissociation (OzID) [19] and Ultra-Violet
 74 Photodissociation (UVPD) [20], but these methods require instrumental modifications. Another
 75 approach involves the derivatization of C=C bonds using Paternò-Büchi reaction, a photochemical
 76 [2+2] cycloaddition between a ketone and an olefin resulting in the formation of an oxetane ring (Fig.
 77 1b) [21]. As demonstrated on phospholipids, this reaction enables the localization of C=C double bonds
 78 through CID fragmentation of the oxetane rings, generating specific retro-Paternò-Büchi fragments
 79 (${}^X\text{F}_\text{O}$ and ${}^X\text{F}_\text{A}$, Fig. 1b). Additionally, Paternò-Büchi derivatization enhances lipid ionization, particularly
 80 when reagents bear a functional group with a high proton affinity [22].



81

82 **Fig. 1** (a) Typical structure of an unsaturated AHL and (b) principle of this AHL characterization by the
83 Paternò-Büchi derivatization. The structure of the two fragments of interest depends on the position
84 of the unsaturation (X) and contains either an aldehyde ($^X\text{F}_A$) or an olefin ($^X\text{F}_O$). The mechanism of the
85 Paternò-Büchi reaction is available in Fig. S1.

86 To our knowledge, none of these methods, including Paternò-Büchi derivatization, has been used on
87 AHLs to localize C=C double bonds on the acyl chain. This study aimed to optimize the Paternò-Büchi
88 derivatization in solution for a better structural characterization of AHLs, especially in determining
89 double bond positions. The optimized method was then applied to highly complex bacterial extracts
90 from several strains of *Burkholderia* and *Paraburkholderia* species to evaluate the matrix influence
91 and as a proof of concept towards the determination of bacterial signatures.

92 2. Material and methods

93 2.1. Chemicals and standards

94 Unless specified, all chemicals used were of analytical grade or high purity. Ultra-pure water was
95 obtained from Direct-Q3[®] water purification system (Merck Millipore, Amsterdam, The Netherlands).
96 Acetonitrile (ACN), methanol (MeOH), isopropanol (IPA) and ethanol (EtOH) were purchased from
97 Carlo Ebra (Val-de-Reuil, France). Technical grade ethyl acetate was purchased from ThermoFisher
98 Scientific (Courtaboeuf, France). Potato dextrose agar was purchased from Condalab (Madrid, Spain).
99 2-acetylpyridine (APy), 2'-4'-5'-trifluoroacetophenone (TriFAP) and *N*-3-oxo-octanoyl-L-homoserine
100 lactone (3-oxo-C8:0-HSL) were purchased from Sigma Aldrich (Saint-Quentin Fallavier, France). *N*-*cis*-
101 octadec-9*Z*-enoyl-L-homoserine lactone (C18:1- Δ 9-*cis*-HSL), *N*-3-oxo-tetradec-7(*Z*)-enoyl-L-
102 homoserine lactone (3-oxo-C14:1- Δ 7-*cis*-HSL), *N*-3-oxo-hexadec-11(*Z*)-enoyl-L-homoserine lactone (3-
103 oxo-C16:1- Δ 11-*cis*-HSL) and *N*-*cis*-tetradec-9*Z*-enoyl-L-homoserine lactone (C14:1- Δ 9-*cis*-HSL) were
104 purchased from Cayman Chemicals Company (Ann Arbor, MI, USA). C18:1- Δ 9-*trans*-HSL, C18:2- Δ 9,12-
105 *cis*-HSL and C18:3- Δ 9,12,15-*cis*-HSL standards were synthesized from L-homoserine lactone, available
106 from L-methionine [23] (Fig. S2, S3 and S4). 3-oxo-C8:0-HSL, C18:1- Δ 9-*cis*-HSL, C18:1- Δ 9-*trans*-HSL,
107 C18:2- Δ 9,12-*cis*-HSL, C18:3- Δ 9,12,15-*cis*-HSL, 3-oxo-C14:1- Δ 7-*cis*-HSL, 3-oxo-C16:1- Δ 11-*cis*-HSL and
108 C14:1- Δ 9-*cis*-HSL were dissolved at 2.5 mM in ACN and store at -20 °C. APy and TriFAP were diluted at
109 0.5 M in MeOH and stored at room temperature.

110 2.2. Biological samples, extraction and sample preparation

111 Two bacterial strains of *Burkholderia* and *Paraburkholderia* genus were used in this study:
112 *Burkholderia* sp. BSNB-0181 and *Paraburkholderia tropica* BSNB-0562 strains isolated from plant and
113 termite holobionts [24, 25] (APA authorization : ABSCH-IRCC-FR-248781-1 and ABSCH-IRCC-FR-
114 248782-1). All strains were maintained at -80 °C in H₂O/glycerol mixture and referenced in the "Strain
115 library" of the Institut de Chimie des Substances Naturelles. Each bacterial strain was cultured on
116 potato dextrose agar in 3 Petri dishes for one week at 28°C. Then, solid-liquid extractions were
117 performed in 200 mL of ethyl acetate while shaking at 80 rpm for 24 hours at room temperature on a
118 TR-250 shaker (Infors HT, Massy, France). The extracts were filtered on paper filters and washed with
119 200 mL of water. The organic phase was evaporated to dryness using a SyncorePlus Analyst evaporator
120 (Büchi, Villebon-sur-Yvette, France), then dissolved in MeOH before being filtered through a 0.2 μm
121 pore-size polytetrafluoroethylene filter (Interchim, Montluçon, France) previously washed with
122 MeOH. Finally, extracts were evaporated to dryness with a Hei-VAP Value rotary evaporator
123 (Heidolph, Modeville, France). An extract of the culture media without microorganisms was also

124 conducted. Final crude extracts were dissolved in MeOH at 10 mg.mL⁻¹, and diluted in ACN to a final
125 concentration of 1 mg.mL⁻¹.

126 2.3. Optimization of the Paternò-Büchi derivatization in solution

127 2.3.1. Paternò-Büchi derivatization

128 For the reaction optimization, several parameters were evaluated through a full-factorial design of
129 experiments (DOE): the solvent (MeOH, EtOH, IPA, ACN), the reagent (APy, TriFAP), the UV wavelength
130 (254 nm, 365 nm) and the AHL/reagent molar ratio (1/50, 1/100).

131 Reaction solutions containing 3-oxo-C8-HSL (100 µM), C18:1-Δ⁹-*cis*-HSL (100 µM) and the reagent (5
132 or 10 mM) were prepared in solvent previously degassed using an Ultrasonic Cleaner USC-T (WVR,
133 Rosny-sous-Bois, France) for 5 minutes to prevent side reactions with dissolved O₂ [26]. Solutions were
134 poured into custom-made quartz tubes (0.4 cm ID, 0.6 cm OD) placed parallel to an Analytica Jena UV
135 Pen-Ray lamp at 254 nm (> 2800 microwatt.cm⁻² at 1" distance) or 365 nm (> 850 microwatt.cm⁻² at
136 1" distance) (respectively 90-0012-01 or 90-0079-01 references from Eurobio Scientific, Les Ulis,
137 France) previously heated for 5 min. The UV lamp was about 1.0-1.5 cm away from the quartz tube.
138 After a 15-minute UV exposure, samples were collected in aluminum foil-covered vials to prevent
139 degradation and diluted in MeOH to a final concentration of 2.5 µM per AHL standard prior LC-MS/MS
140 analysis.

141 2.3.2. Kinetic study

142 A kinetic study was performed on C18:1-Δ⁹-*cis*-HSL using optimized solvent, reagent, UV wavelength
143 and AHL/reagent ratio to optimize the reaction time. Samples were prepared in triplicate and collected
144 at different UV-exposure times between 0 s and 60 min. Samples were poured in aluminum foil-
145 covered vials and diluted at 2.5 µM in methanol prior LC-MS/MS analysis.

146 2.3.3. Stability study

147 Two stability studies were performed to assess storage conditions: one at 6 °C and the other with
148 freeze/thaw (-20 °C/6 °C) cycles. For the 6 °C stability, samples underwent repeated LC-MS/MS
149 analyses for 38 h to simulate extended analysis sequence. For the freeze/thaw stability, samples
150 underwent several-day storage at -20 °C, followed by a 2-hour thawing period at 6 °C before being re-
151 stocked at -20 °C, mimicking samples requiring multiple re-analyses. All samples, derivatized in
152 triplicate under optimized conditions, were collected in aluminum foil-covered vials and diluted at
153 2.5 µM in MeOH.

154 2.3.4. Application to *Z/E* stereoisomers and AHLs containing several double bonds

155 Paternò-Büchi reactions were performed in solution on C18:1-Δ⁹-*trans*-HSL, C18:1-Δ^{9,12}-*cis*-HSL and
156 C18:1-Δ^{9,12,15}-*cis*-HSL at 100 µM to ensure that the optimized conditions would also be successful
157 on AHLs with multiple unsaturations and both *Z/E* configuration.

158 2.4. Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

159 LC-MS/MS analyses were performed on a 1260 Infinity II liquid chromatography (Agilent Technologies,
160 Massy, France) hyphenated to a 6546 Q-TOF mass spectrometer (Agilent Technologies, Massy, France)
161 equipped with a Dual Jet ESI ion source (Agilent Technologies, Massy, France) and operated by
162 MassHunter software (Agilent Technologies, Massy, France). A volume of 5 µL of standard solution or

163 bacterial extracts at 1 mg.mL⁻¹ was injected on a Accucore RP column (100 mm × 2.1 mm, 2.6 μm)
 164 from Thermo Fisher Scientific (Courtaboeuf, France) heated at 50 °C. A mobile phase gradient with
 165 0.1 % formic acid in ultrapure water (A) and acetonitrile (B) was used at a flow rate of 0.4 mL.min⁻¹.
 166 For the optimization of the Paternò-Büchi conditions, the following gradient was used: 0.0-1.0 min
 167 (30-75 %B), 1.0-3.0 min (75-80 %B), 3.0-3.5 min (80-100 %B), 3.5-7.0 min (100 %B), 7.0-7.5 min (100-
 168 30 %B) and 7.5-10.0 min (30 %B). For bacterial extracts analysis, the following gradient was used: 0.0-
 169 12.0 min (5-100 %B), 12.0-16.0 min (100 %B), 16.0-16.5 min (100-5 %B) and 16.5-20.0 min (5 %B). MS
 170 was performed in ion positive mode over the 90-1700 *m/z* range, with a scan rate of 10 spectra/s.
 171 Instrument parameters were set as follows: drying gas at 325 °C and 10 L.min⁻¹, nebulizer at 30 psi,
 172 sheath gas at 350 °C and 10 L.min⁻¹, capillary voltage at 3500 V, nozzle voltage at 500 V, fragmentor
 173 at 110 V, skimmer at 45 V and octopole RF frequency at 750 Hz. For the optimization of the Paternò-
 174 Büchi conditions, targeted MS/MS using CID fragmentation mode was performed with a MS/MS rate
 175 of 4 spectra/sec and a collision energy fixed at 20 eV. For the assessment of the matrix effect and for
 176 the analyses of bacterial extracts, data-dependent MS/MS was performed using CID fragmentation
 177 mode with a collision energy fixed at 20 eV, a maximum 4 precursors per cycle at a MS/MS rate of
 178 4 spectra/sec, an isolation window of 1.3 amu and an active exclusion of 0.02 min after 1 spectrum.

179 2.5. Application to bacterial extracts

180 2.5.1. Estimation of the AHL content

181 Prior derivatization, AHLs from bacterial extracts at 1 mg.mL⁻¹ were quickly annotated from LC-MS/MS
 182 analyses. Data processing was performed with MZmine 3 [27] and a molecular network was then
 183 calculated and visualized using MetGem software (version 1.4.1) [28, 29]. All parameters used for both
 184 software are detailed in Supplementary Information (section 3). The AHLs cluster was localized from
 185 the loss of the lactone moiety (product ion at *m/z* 102.0550) [30]. AHLs were annotated from this
 186 cluster by comparison of experimental [M_{AHL}+H]⁺ *m/z* values with a homemade database (Table S1)
 187 and retention times (when standards were available). The overall AHL concentration was roughly
 188 estimated from LC-MS/MS analyses.

189 2.5.2. Adaptation of the Paternò-Büchi derivatization to the bacterial extracts matrix

190 The matrix effect on the ionization efficiency and on the Paternò-Büchi derivatization were assessed
 191 following the methods developed by Rebane *et al.* [31]. Briefly, two spiking methods were employed
 192 to evaluate the ESI matrix effect and the derivatization matrix effect simultaneously. On one hand, the
 193 overall matrix effect (*ME*%^{*}) was determined using the post-extraction spike method, where a
 194 derivatized standard solution is compared with a bacterial extract spiked with standards before
 195 derivatization (Equation 1). On the other hand, the ESI matrix effect (*dME*%) was determined using
 196 the post-derivatization spike method, where a derivatized standard solution is compared with a
 197 derivatized bacterial extract spiked with derivatized standards (Equation 2). The relative yield of the
 198 Paternò-Büchi derivatization (*Y*_{rel}^{PB}) was obtained from Equation 3.

$$199 \quad ME\%^* = \frac{A_{matrix}^*}{A_{STD}} * 100 \quad (1)$$

$$200 \quad dME\% = \frac{A_{matrix}}{A_{STD}} * 100 \quad (2)$$

$$201 \quad Y_{rel}^{PB} = \frac{\%ME^*}{\%dME} * 100 \quad (3)$$

202 With A_{matrix}^* and A_{matrix} the LC-MS peak areas of the Paternò-Büchi products respectively from the
203 post-extraction spike and the post-derivatization spike methods, A_{STD} the peak area of the Paternò-
204 Büchi products from the standard solution.

205 Six AHLs (C14:1- Δ^9 -*cis*-HSL, 3-oxo-C14:1- Δ^7 -*cis*-HSL, 3-oxo-C16:1- Δ^{11} -*cis*-HSL, C18:1- Δ^9 -*cis*-HSL,
206 C18:2- $\Delta^9,12$ -*cis*-HSL and C18:3- $\Delta^9,12,15$ -*cis*-HSL) were mixed in the spiking solution. Several
207 combinations of AHL concentrations (2.5 μ M and 25 μ M for each AHL) and APy concentrations
208 (1.5 mM, 15 mM and 150 mM) were used for Paternò-Büchi derivatizations. Prior LC-MS/MS analysis,
209 all solutions were diluted to the same concentration of underivatized analytes. The bacterial extract
210 used for this study was from the *Burkholderia* sp. BSNB-0181 strain as no unsaturated AHL was
211 detected by LC-MS/MS.

212 Derivatization of the *Paraburkholderia tropica* BSNB-0562 strain bacterial extract (1 mg.mL⁻¹) was
213 performed in newly optimized conditions. After derivatization, samples were collected in aluminum
214 foil-covered vials and directly analyzed by LC-MS/MS.

215 2.6.3. Localization of AHL double bonds

216 LC-MS/MS data of derivatized *Paraburkholderia tropica* bacterial extract were manually interpreted
217 using Qualitative Mass Hunter (Agilent Technologies, Massy, France). The focus was put on the
218 unsaturated AHLs previously annotated in the molecular network. The identification of the Paternò-
219 Büchi products was based on the exact $[M_{AHL}+M_{reagent}+H]^+$ m/z values. The first double bond was
220 localized from the theoretical fragments of the oxetane rings [22] (Table S2), and in case of several
221 double bonds, the other positions were determined based on the difference of 40 amu between
222 fragments of the same series.

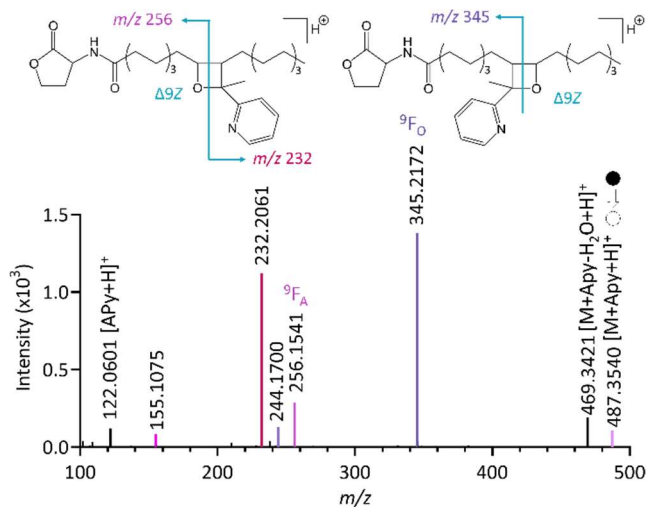
223 3. Results and discussion

224 3.1. Localization of the C=C bonds

225 Before optimizing the derivatization conditions, it was crucial to check the successful CID
226 fragmentation of the oxetane rings for the two Paternò-Büchi products with both 2',4',5'-
227 trifluoroacetophenone (TriFAP) and 2-acetylpyridine (APy). Theoretically, CID fragmentation will lead
228 to two specific fragments containing the homoserine lactone moiety, $^X F_O$ and $^X F_A$ arising from each
229 regioisomer of the Paternò-Büchi products (where X corresponds to the double bond position in the
230 initial AHL, fragment F_O contains an olefin, and fragment F_A contains an aldehyde) (Fig. 1b). [21]

231 When using TriFAP as a reagent in presence of C18:1- Δ^9 -*cis*-HSL, the Paternò-Büchi products were
232 detected at m/z_{theo} 540.3295 as expected, but at a very low intensity. Nevertheless, no theoretical
233 oxetane ring fragments (m/z_{theo} 398.1938 and m/z_{theo} 256.1543) [22] were detected after CID
234 experiments (Fig. S5). This may be due to the low ionization efficiency of the TriFAP-derived
235 fragment. On the other hand, APy allowed to generate the Paternò-Büchi products of C18:1- Δ^9 -*cis*-HSL
236 at m/z_{exp} 487.3540 (Δ_{error} = 2.1 ppm) and the characteristic CID fragments $^9 F_A$ and $^9 F_O$ at m/z_{exp} 256.1541
237 (Δ_{error} = 0.8 ppm) and m/z_{exp} 345.2172 (Δ_{error} = 0.3 ppm), respectively (Fig. 2). Moreover, the
238 complementary fragment ions at m/z_{exp} 232.2061 (Δ_{error} = 0.4 ppm) and m/z_{exp} 244.1700 (Δ_{error} = 1.6
239 ppm) were also detected confirming the unambiguous C=C localization. The ion at m/z_{exp} 155.1075
240 (Δ_{error} = 5.2 ppm) is coming from the ion at m/z_{exp} 256.1541 (Δ_{error} = 0.8 ppm) resulting from the
241 fragmentation of the amide bond whereas m/z_{exp} 122.0601 (Δ_{error} = 0.8 ppm) is originating from a
242 gas-phase retro-Paternò-Büchi reaction leading to the formation of the protonated APy. Fragmentation
243 mechanisms of both regioisomers are available in Fig. S6 and the energy resolved mass spectrometry

244 breakdown curve of the C18:1- Δ^9 -*cis*-HSL Paternò-Büchi products is available in Fig. S7 allowing to
245 determine the optimized CID energy for the structural characterization of derivatized C18 AHLs.
246 Thus only APy will be employed as reagent for the next experiments.

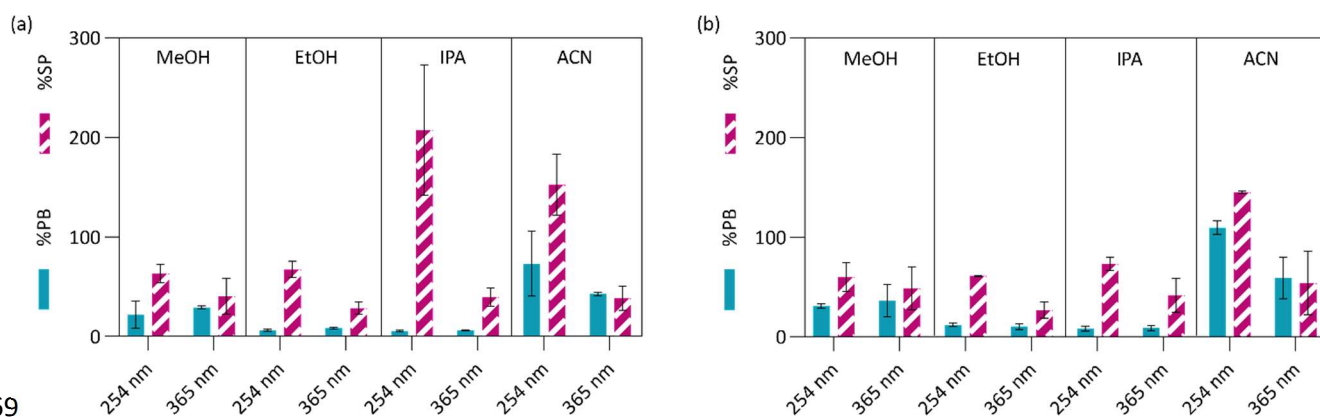


247
248 **Fig. 2** CID-MS/MS spectrum of the Paternò-Büchi products (m/z_{exp} 487.3540, $\Delta_{\text{error}} = 2.1$ ppm) of C18:1-
249 Δ^9 -*cis*-HSL (derivatization at 100 μM and analysis at 2.5 μM) after the in-solution derivatization using
250 APy as reagent in acetonitrile after a 15-minute exposure at 254 nm. The MS/MS spectrum was
251 obtained with a collision energy set at 15 eV.

252 3.2. Optimization of the Paternò-Büchi reaction in solution from the full factorial DOE

253 To assess the efficiency of Paternò-Büchi derivatization for AHLs, two key factors were considered:
254 maximizing product intensity to achieve high-quality MS/MS spectra and minimizing potential side
255 reactions. Relative amounts of Paternò-Büchi products ($\%^{\text{PB}}$) and side products ($\%^{\text{SP}}$) were evaluated
256 to determine optimal conditions. Details on the calculation of $\%^{\text{SP}}$ and $\%^{\text{PB}}$ can be found in the
257 supplementary information (section 5 and Fig. S8 to S10). A full factorial DOE was used to optimize the
258 Paternò-Büchi conditions as it enables the simultaneous assessment of several parameters. As shown
259 in Pareto charts available in Fig. S11, it was essential to take into account all parameters and parameter
260 interactions for the optimization of the Paternò-Büchi derivatization. First, as previously explained
261 (section 3.1), APy ($\lambda_{\text{max}1}=227$ nm, $\lambda_{\text{max}2}=267$ nm, Fig. S12) was undoubtedly the best reagent to localize
262 the double bonds from AHL Paternò-Büchi products. As shown in Fig. 3, Paternò-Büchi products were
263 obtained with APy, irrespective of the used AHL/reagent molar ratio, solvent or UV wavelength. It is
264 worth noting that acetone is still the most commonly used reagent for the Paternò-Büchi reaction on
265 other lipids [21, 32–34] even if other reagents [21, 22, 35–38] have also been used. However, acetone
266 was not included as a reagent in our DOE as preliminary results showed no Paterno Buchi products
267 between AHLs and acetone (Fig. S13).

268



269
 270 **Fig. 3** Relative amount of Paternò-Büchi products and side products after a 15-minute derivatization of C18:1- Δ^9 -*cis*-HSL in
 271 various solvents (methanol, ethanol, isopropanol and acetonitrile), using two UV lamps at different wavelengths (254 nm
 272 and 365 nm) and acetylpyridine at a AHL/reagent ratio of (a) 1/50 and (b) 1/100. Reactions were performed in triplicates
 273 (n=3) and samples were analyzed by LC-MS/MS.

274 As shown in Fig. 3, compared to protic solvents, acetonitrile yielded a higher quantity of Paternò-Büchi
 275 products due to the lower energy requirement for the carbonyl $n \rightarrow \pi^*$ transition [39]. The choice of
 276 solvent and UV wavelength are correlated, with the optimal UV wavelength differing depending on
 277 the solvent. Acetonitrile at 254 nm was finally chosen to optimize the detection of Paternò-Büchi
 278 products. This result is consistent with previous finding on lipids from human plasma [37].

279 The AHL/APy molar ratio of 1/100 was determined as optimal, showing increased detection of
 280 Paternò-Büchi products while maintaining consistent side product levels across ratios of 1/20 to 1/200
 281 (Fig. S14). However, beyond the 1/100 ratio, improvements in Paternò-Büchi products detection were
 282 not significant. Therefore, the optimal AHL/APy ratio was set at 1/100 to prevent source
 283 contamination with excess of APy.

284 3.3. Kinetic study

285 The optimal reaction time for maximizing Paternò-Büchi derivatization was determined through a
 286 kinetic study of the reaction between C18:1- Δ^9 -*cis*-HSL (100 μ M during the derivatization and 2.5 μ M
 287 during the LC-MS/MS analysis) and APy (Fig. S15). %PB increased over time, peaking at 15 min, before
 288 decreasing due to the formation of a higher proportion of unknown side products and potential retro-
 289 Paternò-Büchi reaction. Thus, a 15-minute derivatization time at 254 nm, in acetonitrile, and with APy
 290 at a AHL/reagent ratio of 1/100 was selected.

291 3.4. Stability studies

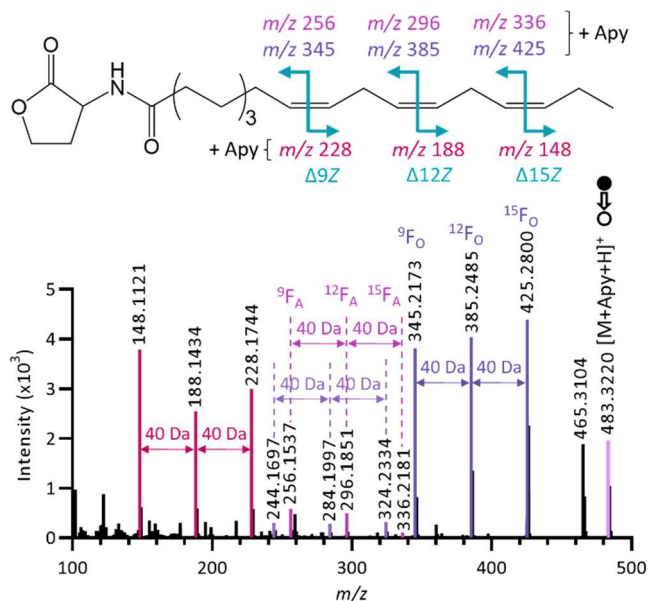
292 No clear degradation of the Paternò-Büchi products or side products was observed during 38 hours of
 293 storage at 6 °C (Fig. S16a) or after several freeze/thaw cycles as well (Fig. S16b). Therefore, derivatized
 294 samples can undergo extended LC sequences or multiple analyses without compromising the results.
 295 To our knowledge, this is the first stability study carried out on Paternò-Büchi products.

296 3.5. Paternò-Büchi derivatization of *Z/E* stereoisomers and AHLs with several double bonds

297 The MS/MS spectra of both *Z* and *E* stereoisomers exhibit similar patterns, indicating identical
 298 fragmentation of the resulting Paternò-Büchi products and ruling out stereochemical characterization
 299 of the double bond by this method (Fig. S17a and Fig. S17b). Additionally, Paternò-Büchi derivatization
 300 enables the localization of up to three double bonds on a single AHL, with no detection of multiple
 301 oxetane ring-containing products (Fig S17a and Fig. 4), suggesting that APy reacted with only one
 302 double bond at a time. Once more, the $^x\text{F}_0$ fragments consistently exhibited the highest intensity

303 across all AHL standards (Fig. 4 and Fig. S17), regardless of double bond position, stereochemistry, or
304 number of unsaturations, thus confirming the annotation rule outlined in section 3.1.

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307 **Fig. 4** CID MS/MS spectra of the Paternò-Büchi products of C18:3-Δ^{9,12,15}-cis-HSL (m/z_{exp} 483.3220, $\Delta_{\text{error}} = 0.0$ ppm) after in-
308 solution derivatization using the APy reagent in acetonitrile after a 15-minute exposure at 254 nm. The MS/MS spectra were
309 obtained with a collision energy set at 15 eV. All remaining fragments are annotated in Fig. S18.

310 3.6. Paternò-Büchi derivatization on bacterial extracts

311 3.6.1. Evaluation of the matrix effect

312 When extracting bacterial cultures with ethyl acetate, AHLs are typically mixed with other unsaturated
313 specialized metabolites, potentially containing double bonds reactive with APy under UV irradiation
314 [40, 41]. Additional experiments were conducted to evaluate the matrix effect on the previously
315 developed method and adjust the AHL/APy molar ratio to account for the presence of other
316 unsaturated compounds. For that purpose, C18:1-Δ⁹-cis-HSL was added to the bacterial extract of the
317 *Burkholderia* sp. BSNB-0181 strain where no unsaturated AHL was detected by LC-MS/MS. Two AHL
318 concentrations were tested (2.5 μM and 25 μM) and three AHL/APy molar ratio were evaluated
319 (1/100, 1/1000 and 1/10000). However, at the lowest AHL and APY concentrations (2.5 μM, 1/100
320 ratio, data not shown), most Paternò-Büchi products were below the limit of detection.

321 With the previous AHL/APy ratio of 1/100, the overall matrix effect induced a loss of at least 83 % of
322 the Paternò-Büchi products peak areas ($ME\%$ * below 17 %, Fig. S19a) while the ESI matrix effect led
323 to a maximum loss of 26 % ($dME\%$ above 74 %, Fig. S19b), resulting in a maximum relative yield Y_{rel}^{PB}
324 of 20 % for all AHL standards (Fig. S19c). Adjusting the APY/AHL ratio to 1/1000 increased the relative
325 yield Y_{rel}^{PB} to between 26 % and 50 % (Fig. S19c), depending on the AHL standard and its initial
326 concentration. However, further increasing the AHL/APy molar ratio to 1/10000 resulted in lower
327 relative yields Y_{rel}^{PB} ranging between 18 % and 33 % (Fig. S19c). Therefore, the AHL/APy molar ratio of
328 1/1000 was selected for the derivatization of bacterial extracts.

329 3.6.2. Identification of AHLs and localization of C=C bonds in bacterial extract

330 As a proof of concept, the optimized Paternò-Büchi conditions were applied on the bacterial extract
331 of the *Paraburkholderia tropica* BSNB-0562 strain. From the analysis of this bacterial extract prior

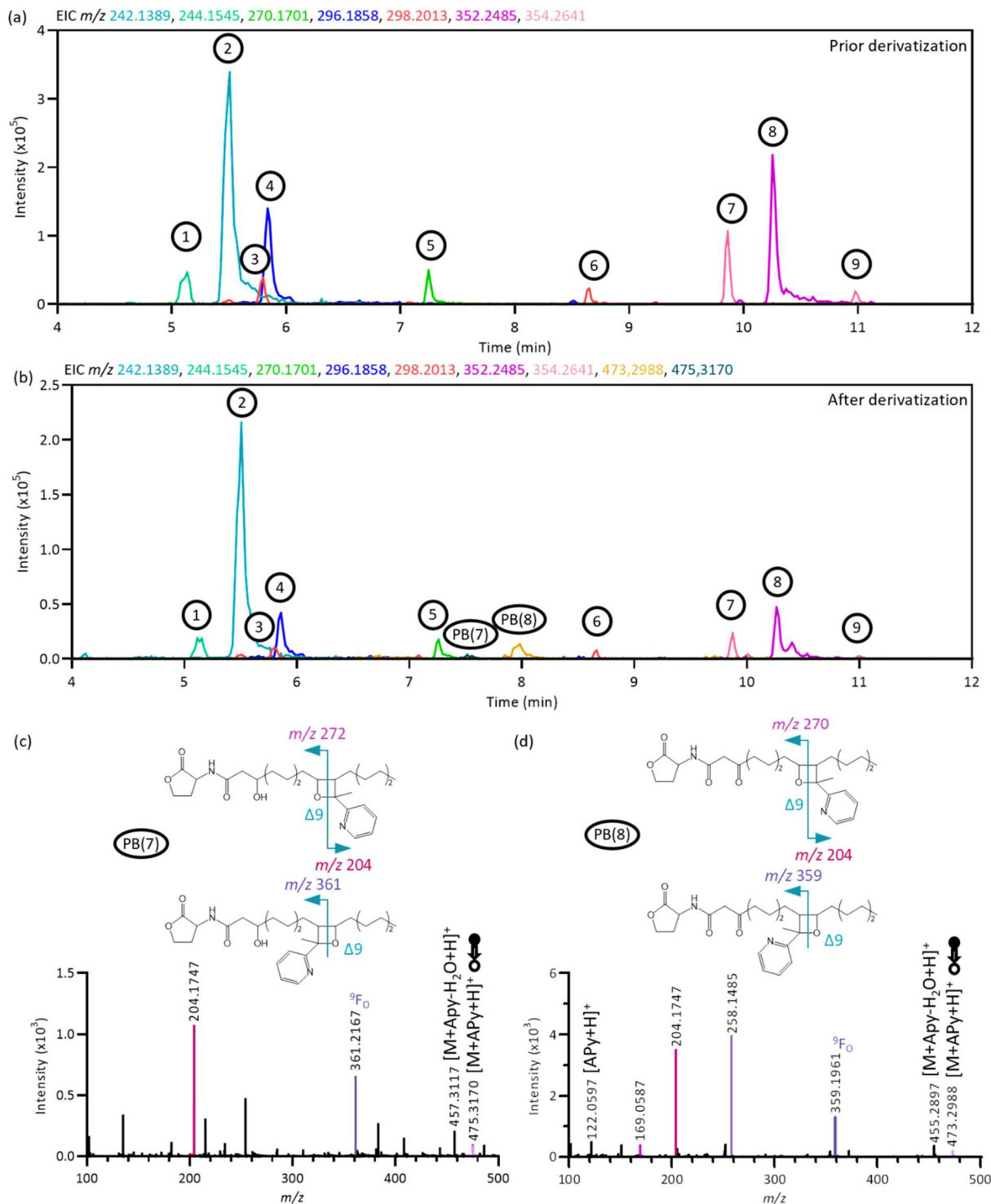
332 derivatization (Fig. 5a), a molecular network containing 411 nodes was generated (Fig. S20). As AHLs
 333 show similar fragmentation patterns including the loss of the lactone moiety (product ion at m/z_{theo}
 334 102.0550) [30], the cluster of AHLs was easily located in the molecular network. From this cluster,
 335 seven AHLs, including two containing double bonds, were annotated (Table 1) by comparison of
 336 experimental $[M_{\text{AHL}} + H]^+$ m/z values with a homemade database (Table S1) and retention times (when
 337 standards were available). Five AHLs were annotated as saturated as they matched with the database
 338 (Table S1).

339 After derivatization, double bonds of these two unsaturated AHLs (compound PB(7) and PB(8) in Fig
 340 5b) were localized from the MS/MS spectra of Paternò-Büchi products, revealing OH-C16:1- Δ^9 -HSL
 341 (compound 7, Fig. 5c) and oxo-C16:1- Δ^9 -HSL (compound 8, Fig. 5d). After derivatization, as the
 342 Paternò-Büchi yield is not 100 %, both underivatized AHLs (compounds 7 and 8) were still detected
 343 (Fig 5b) and showed peak duplication. This duplication suggests the *Z/E* isomerization of C=C bonds
 344 during the UV exposure. The same trend was also observed when C18:1- Δ^9 -*cis*-HSL and C18:1- Δ^9 -
 345 *trans*-HSL standards were derivatized (Fig S10), making it easier to detect AHLs containing double
 346 bonds.

347 The last two unknown AHLs were detected at 298.2016 (compound 3) and 296.1858 (compound 4).
 348 By comparison with the database (Table S1), compound 3 could be oxo-C12:1-HSL or OH-C12:2-HSL
 349 based on the $[M+H]^+$ m/z and compound 4 could be OH-C12:1-HSL based on the $[M+H]^+$ m/z . However,
 350 these two AHLs neither reacted with APy, nor showed *Z/E* isomerization (no duplication of peaks 3
 351 and 4 in Fig 5b). This raised the question of their chemical structure, indicating that they do not contain
 352 any C=C double bond or that this double bond has a different reactivity towards the Paternò-Büchi
 353 and *Z/E* isomerization reactions in these optimized conditions (it may be an enone). Another
 354 hypothesis could be the presence of cyclic carbon-chains on AHLs. Even if it seems to be rare, unusual
 355 classes of AHL (such as coumaroyl-HSL) were previously reported [42]. Further investigations,
 356 including isolation, purification and full structural characterization by 2D-RMN, should be performed
 357 to conclude.

358 **Table 1** Annotated AHLs produced by the *Paraburkholderia tropica* BSNB-0562 strain. *Compounds 3 and 4 are
 359 annotated as unknown AHL as they have the same $[M+H]^+$ m/z as respectively oxo-C12:1-HSL/OH-C12:2-HSL and
 360 OH-C12:1-HSL, but did not react with APy (no Paternò-Büchi products or peak duplication observed). One
 361 hypothesis is that these AHLs could be an enone or cyclic AHL.

Peak number	Annotated AHLs	Molecular formula	Theoretical $[M+H]^+$ m/z	Observed $[M+H]^+$ m/z	Δ_{error} (ppm)	Retention time (min)
1	OH-C8:0-HSL	C ₁₂ H ₂₁ NO ₄	244.1543	244.1543	0.0	5.14
2	oxo-C8:0-HSL	C ₁₂ H ₁₉ NO ₄	242.1387	242.1388	0.4	5.51
3	Unknown AHL*	C ₁₆ H ₂₇ NO ₄	298.2013	298.2016	0.1	5.80
4	Unknown AHL*	C ₁₆ H ₂₅ NO ₄	296.1856	296.1858	0.7	5.86
5	oxo-C10:0-HSL	C ₁₄ H ₂₃ NO ₄	270.1700	270.1704	1.5	7.25
PB(7)	Paternò-Büchi products of OH-C16:1- Δ^9 -HSL	C ₂₇ H ₄₂ N ₂ O ₅	475.3166	475.3170	0.8	7.98
PB(8)	Paternò-Büchi products of oxo-C16:1- Δ^9 -HSL	C ₂₇ H ₄₀ N ₂ O ₅	473.3010	473.2988	4.6	7.52
6	oxo-C12:0-HSL	C ₁₆ H ₂₇ NO ₄	298.2013	298.2013	0.0	8.65
7	OH-C16:1- Δ^9 -HSL	C ₂₀ H ₃₅ NO ₄	354.2639	354.2645	1.7	9.62
8	oxo-C16:1- Δ^9 -HSL	C ₂₀ H ₃₃ NO ₄	352.2482	352.2488	1.7	10.27
9	oxo-C16:0-HSL	C ₂₀ H ₃₅ NO ₄	354.2639	354.2639	0.0	10.98



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Fig. 5 Extracted ion chromatograms of all AHLs detected (a) prior derivatization and (b) after derivatization in the bacterial extract from the *Paraburkholderia tropica* BSNB-0562 strain (see Table 1 for peak identity). MS/MS spectra of the Paternò-Büchi products of (c) OH-C16:1- Δ 9-HSL and (d) oxo-C16:1- Δ 9-HSL. All remaining fragments are annotated in Fig. S21. The Paternò-Büchi derivatization was performed in acetonitrile using Apy as reagent at AHL/Apy molar ratio of 1/1000 during a 15-minute UV exposure at 254 nm.

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The results obtained for this bacterial extract show that it is possible to use our optimized Paternò-Büchi conditions to localize the AHLs double bonds after the derivatization of the bacterial extract. This method could be used on other bacterial extracts of different strains or bacterial species.

373 4. Conclusion

374 We first optimized a method for the fine structural determination of AHLs, in particular to localize C=C
375 bonds using Paternò-Büchi reaction in solution, directly from a diluted bacterial extract. In terms of
376 analytical developments, it has been demonstrated that the Paternò-Büchi derivatization can be
377 performed post-LC column on unsaturated phospholipids [43]. This method could then be
378 implemented to characterize unsaturated AHLs in complex extracts taking into account that a large
379 excess of APy is required (optimized to avoid as much as possible instrument contamination by a basic
380 compound). For such post-LC column method, the reaction time could be lowered by optimizing the
381 UV-transparent material used between the LC column to the MS. As AHLs are involved in bacterial
382 communication and virulence, *in-situ* imaging on Petri dishes or biofilms on diverse surfaces after
383 derivatization can be envisaged, as already described for phospholipids in biological tissues [44]. This
384 may be particularly relevant as a diagnostic mean to detect molecular signatures in infectious
385 bacteriology. On a more fundamental point of view, this method could be extended to tracking
386 unsaturated long chains in metabolites.

387 5. CRediT authorship contribution statement

388 **Clarisse Gosset-Erard:** Methodology, Validation, Formal analysis, Investigation, Writing – original
389 draft, Visualization. **Guanghui Han:** Resources, Writing – review & editing. **Dimitra Kyrko:**
390 Investigation, Writing – review & editing. **Amandine Hueber:** Investigation, Supervision, Writing –
391 review & editing. **Bastien Nay:** Conceptualization, Resources, Methodology, Writing – review &
392 editing, Funding acquisition. **Véronique Eparvier:** Conceptualization, Resources, Methodology,
393 Writing – review & editing, Funding acquisition. **David Touboul:** Conceptualization, Methodology,
394 Writing – review & editing, Funding acquisition, Supervision.

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400 used for preliminary analyses and the LAMBE laboratory for the access to the UV-vis
401 spectrophotometer to acquire UV spectra of APy. The graphical abstract was created with
402 Biorender.com.

403 7. Data availability

404 The datasets generated and analyzed during the current study are available from the corresponding
405 author on request.

406 8. Conflicts of Interest

407 The authors declare that they have no known competing interests or personal relationships that could
408 have appeared to influence the work reported in this paper.

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