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Short communication

Rapid identification of 4-hydroxy-2-alkylquinolines produced by *Pseudomonas aeruginosa* using gas chromatography–electron-capture mass spectrometry

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Abstract

The 4-hydroxy-2-alkylquinolines and their N-oxides are secondary metabolites produced by *Pseudomonas aeruginosa* which inhibit the growth of a number of Gram-positive organisms including *Staphylococcus aureus*. To facilitate the identification of these compounds in biological fluids, we have developed a rapid profiling system based on gas chromatography–electron-capture mass spectrometry of the O-bistrifluoromethylbenzoyl derivatives. Using the technique, over twenty hydroxyalkylquinolines have been identified from a culture obtained from a strain of *P. aeruginosa* obtained from a patient with severe bronchiectasis.

1. Introduction

Pseudomonas aeruginosa is an opportunist pathogen which frequently colonises the lungs of patients with bronchiectasis and cystic fibrosis. Colonisation by this organism is associated with a marked deterioration in respiratory function. *P. aeruginosa* secretes a number of low-molecular-mass hydrophobic toxins, many of which contribute to its persistence in human lung, and may be responsible for many of the clinical effects associated with pulmonary colonisation by the organism [1–3]. One group of these pseudomonas secondary metabolites are the 4-hydroxy-2-alkylquinolines and their N-oxides [4]. These compounds have antibiotic activity [5,6] and have recently been shown to be active against most strains of *Staphylococcus aureus* including those resistant to methicillin [7]. Using gas chromatography (GC) together with electron-impact mass spectrometry (EIMS) and fast-atom bombardment mass spectrometry (FAB-MS) it was possible to identify 4-hydroxy-2-heptylquinoline in bronchial secretions of a patient with cystic fibrosis [7]. The formation of these substituted quinolines may account for the inverse correlation between the presence of *P. aeruginosa* and *S. aureus* in the sputum of patients with cystic fibrosis [8]. We have previously shown that a strain of *P. aeruginosa*

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(P3940) can produce heptyl, heptenyl, nonyl, nonenyl, undecyl and undecenyl hydroxyquinolines (and their N-oxides) using HPLC and FAB-MS [7]. We have now extended this work using GC–electron-capture MS to identify a number of hydroxyalkylquinolines (and their alkenyl analogues) produced by two strains of *P. aeruginosa* obtained from patients colonised with this organism.

2. Experimental

All reagents were of AnalaR grade (BDH Chemical Co, Poole, UK) unless otherwise stated. Bistrifluoromethylbenzoyl chloride was obtained from Fluorochem (Glossop, UK). Titanium trichloride, diisopropylethylamine, bistrifluoromethylsilyl–trifluoroacetamide, trimethylchlorosilane and 4-hydroxy-2-heptylquinoline-N-oxide were obtained from Sigma (Poole, UK).

A non-pigmented strain of *P. aeruginosa* (strain P3940) [8] was obtained from a colonised patient with severe bronchiectasis attending a clinic at the Royal Brompton Hospital; it was grown on Kings "A" agar at 37°C for 24 h. The bacterial growth was removed and the agar was cut into small pieces and extracted with chloroform (3 × 50 ml per plate). The chloroform layer was loaded onto silica Sep-Pak cartridges (Waters Ass., Cheshire, UK); following washing with chloroform, the quinolines were eluted with methanol. A second strain of *P. aeruginosa* (PYO5, obtained at the Hammersmith Hospital) was grown on blood agar plates and incubated at 37°C overnight. This strain produced the blue pigment, pyocyanin. The bacteria were removed by scraping and resuspended in brain heart infusion (BHI) broth (Sigma). A heavy suspension was then used to inoculate 400 ml of BHI and incubated at 37°C overnight. The culture was centrifuged at 500 *g* for 15 min, and the supernatant extracted twice with 2 volumes of chloroform. This preparation was used without Sep-Pak purification. Chemical reduction of authentic 4-hydroxy-2-heptyl-quinoline-N-oxide and the pseudomonas extract was carried out with titanium trichloride (1.9 *M*) in hydrochloric acid (2 *M*) for 30 min at room temperature; the sample was first dissolved in a minimum volume of methanol. Excess water was added to the mixture, and the reduced quinoline extracted twice with 2 volumes of chloroform.

Gas chromatography (GC) was carried out on a DB5 capillary column (30 m × 0.25 mm I.D., 0.25 μm film thickness, Jones Chromatography, Hengoed, UK) using helium as carrier gas. After 1 min at 125°C the column temperature was raised by 20°C/min from 125°C to 325°C. Samples were injected in nonane using a Grob injector in the splitless mode set at 250°C. The GC column was routed into a Finnigan 4500 mass spectrometer (MS) operated either in the electron-impact mode (70 eV electron energy) or under electron-capture conditions (100 eV) with ammonia (0.4 Torr) as reagent gas. Derivatisation was undertaken with either a mixture of bistrifluoromethylsilyl–trifluoroacetamide and trimethylchlorosilane (90:10, v/v) at 60°C overnight, or by treatment with a 5% solution in dry ethyl acetate of a mixture of diisopropylethylamine and bistrifluoromethylbenzoyl chloride (1:1, v/v) for 1 h at room temperature.

3. Results

Cultures of *P. aeruginosa* produce a series of 4-hydroxy-2-alkyl-quinolines (HAQ) and their respective N-oxides. The N-oxides are unstable in solution, slowly converting to the parent quinoline. This reduction occurs rapidly and quantitatively in the presence of $TiCl_3$ [7]. Both the HAQs and their N-oxides have poor gas chromatographic properties; for example, 4-hydroxy-2-heptylquinoline (prepared by $TiCl_3$ reduction of the commercially available N-oxide) runs on GC on a DB5 column with a pronounced leading edge which increased markedly with only a small increase in column loading. Although the O-trimethylsilyl derivative has good chromatographic properties, the silylation reaction was not quantitative.

In contrast, 4-hydroxy-2-heptylquinoline was converted quantitatively to its bistrifl-

uoromethylbenzoate derivative. This derivative eluted from a DB5 GC column as a single symmetrical peak at 361s and generated an intense electron-capture mass spectrum. The majority of the ion current was carried by molecular anion (M^-, m/z 483), which was the base peak of the spectrum. With a signal-to-noise ratio of >10, less than 1 pg of this derivative could be detected in the selected-ion monitoring mode. The N-oxide also formed the bistrifluoromethylbenzoate derivative, however it eluted as a single broad peak on GC later than the corresponding quinoline. The mass spectrum was more complex containing ions at m/z 498 ($M-H^-$) and 481 ($M^- - H_2O$); ions were also observed at m/z 483 and 516 arising from auto-oxidation within the ion source. Clearly, the bistrifluoromethylbenzoyl derivative of the N-oxide is unsuitable for routine analysis. Reduction to the parent hydroxyheptylquinoline is quantitative and rapid, and was used to reduce any N-oxides present in the pseudomonas preparations.

The extracted agar plate preparation from the *P. aeruginosa* strain P3940 was reduced with $TiCl_3$ and derivatised in a similar manner for analysis by GC–ECMS. A number of peaks were observed in the GC profile corresponding to molecular anion (M^-) from a series of alkylhydroxyquinoline bistrifluoromethylbenzoates (Fig. 1). The relative abundance of each substituted quinoline could be estimated from the respective peak areas (Fig. 2a). The heptyl quinoline (C_7-HAQ, m/z 483, 361s) was the most abundant species present, together with appreciable amounts of C_9-HAQ (m/z 511, 412s). Smaller amounts of C_1–C_6-, C_8-, C_{10}-, C_{11}- and C_{13}-HAQs were also observed with

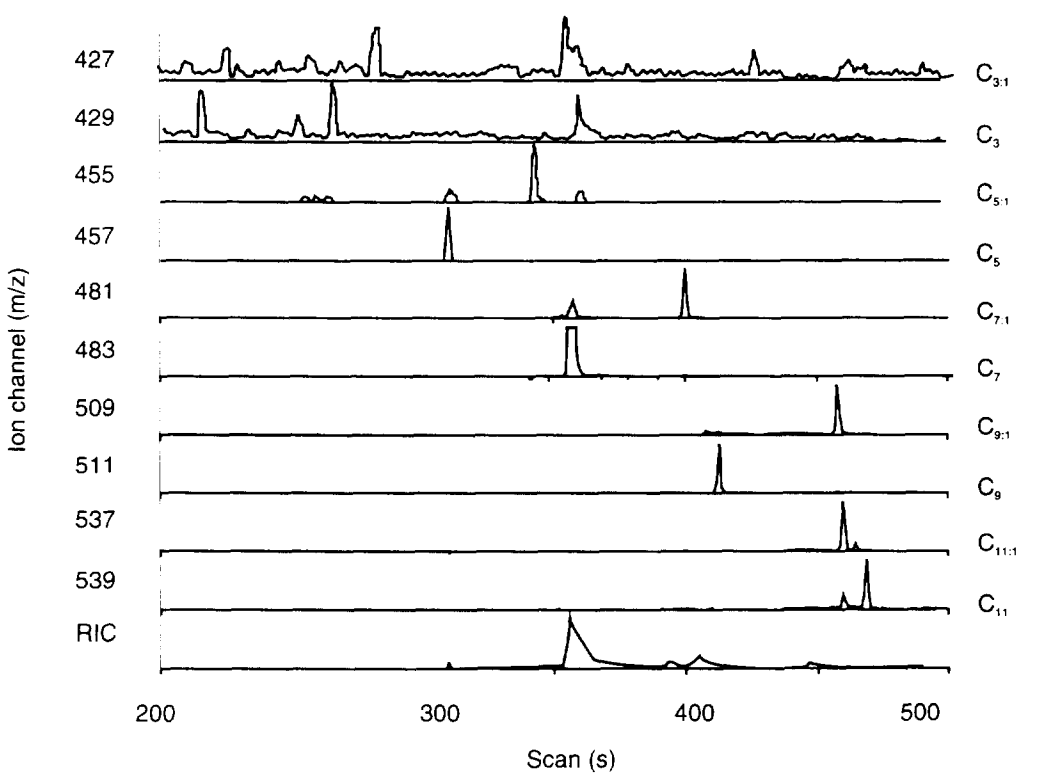

Fig. 1. GC–ECMS ion profile of the molecular anions (M^-) of the odd alkyl chain length HAQs from a strain of *P. aeruginosa* (P3940) obtained from a patient with bronchiectasis. C_7-HAQ (M^- m/z 483) is the major species present, together with appreciable amounts of other analogues.

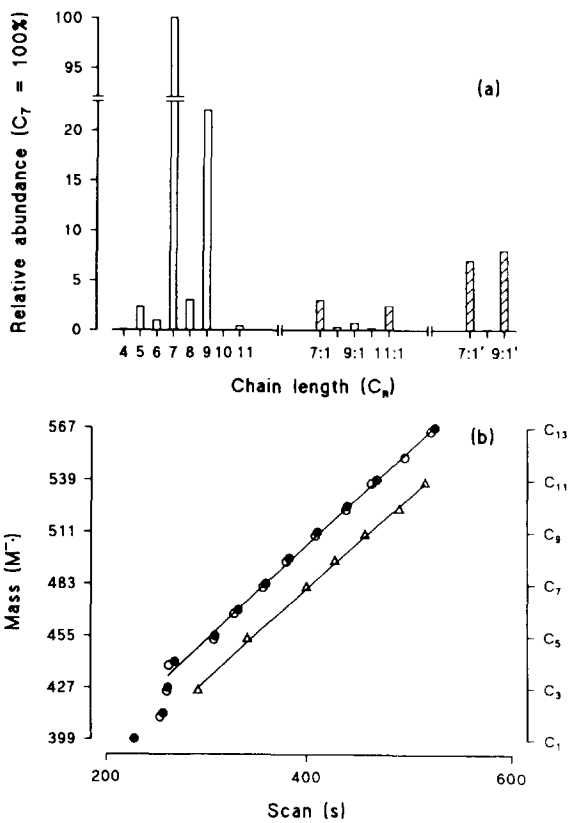

Fig. 2. (a) Relative abundance of the major HAQs present in a pseudomonas culture filtrate. Five further saturated HAQs (C_1–C_4 and C_{13}) were detected with abundances $<0.2\%$ of C_7-HAQ. Eleven other low abundance ($<0.1\%$) unsaturated HAQs were also detected: $C_{2:1}$–$C_{6:1}$-HAQs, $C_{12:1}$-HAQ, $C_{13:1}$-HAQ, $C_{3:1'}$-HAQ, $C_{5:1}$-HAQ, $C_{10:1}$-HAQ and $C_{11:1'}$-HAQ. Identification was facilitated by the linear relationship between alk(en)yl chain length and retention time (Fig. 2b). (b) Correlation between GC retention time on a DB5 column and mass (M) of the derivatised HAQs present in a pseudomonas preparation. Saturated HAQs (C_1–C_{13}) are shown as (●). There are two series of monounsaturated HAQs: $C_{2:1}$–$C_{13:1}$ (○) and $C_{3:1'}$, $C_{5:1'}$, $C_{7:1'}$–$C_{11:1'}$ (△).

molecular ions at m/z 399, 413, 427, 441, 455, 469, 497, 511 and 529, respectively; to obtain mass spectra on the least abundant species it was necessary to increase the column loading such that the C_7-HAQ peak was saturated. There was a linear relationship between GC retention time and chain length for the saturated HAQs (Fig. 2b), although for C_2- and C_1-HAQs, this relationship was not followed. Two series of unsaturated analogues were also present: one series ($C_{n:1}$) eluted 2–3 s before the parent quinoline and a second series ($C_{n:1'}$) eluted 30–50 s after the parent quinoline. The major unsaturated species were the $C_{7:1'}$ (m/z 481, 399s) and $C_{9:1'}$ (m/z 509, 457s) accounting for 7 and 8% respectively of the amount of the most abundant C_7-HAQ (Fig. 2a). The $C_{n:1}$ series followed the same linear relationship for chain length and GC retention time as the saturated analogues, whereas the $C_{n:1'}$ series followed a parallel linear relationship. Nonyl, nonenyl and heptyl HAQs were also readily identified by GC–ECMS in a second pseudomonas extract, PYO5 (data not shown). In both cases, a number of even mass substances were also observed in the GC–ECMS profile, however these did not directly interfere with the identification of the alkyl HAQs.

4. Discussion

P. aeruginosa is an opportunist pathogen which does not affect the lungs of normal people. In patients with cystic fibrosis and other forms of bronchiectasis, and in patients who are ventilated, the lung defences are compromised and the organism can colonise the airways. A number of toxic secondary metabolites facilitate its survival with consequent deleterious effects on the host. The 4-hydroxy-2-alkylquinolines and their N-oxides are one such family of toxins; they are biosynthesised from anthranillic acid and acetyl and malonyl CoA [9]. A number of 4-hydroxy-2-alkylquinolines have been reported [4–7,10], including a ring methylated (3-methyl-2-heptenyl-) analogue [11]. Only 4-hydroxy-2-heptylquinoline has been identified in human samples [7].

To identify these compounds in pseudomonas culture filtrates, we have developed a simple and sensitive method based on GC–ECMS of the bistrifluoromethylbenzoyl derivative. This derivative has been used successfully for many years for the identification and quantitation of a range of hydroxylated and nitrogenous compounds including paracetamol, histamine metabolites and steroids [12–14]. The detection limit is

less than 1 pg on column. We had previously shown using HPLC and fast-atom bombardment mass spectrometry that the pseudomonas extracts contained a number of hydroxy-alkyl and alkenyl quinolines [7]. Using GC–ECMS we have now identified 13 saturated quinolines produced by a strain of *P. aeruginosa* obtained from a patient with bronchiectasis; this includes a number of even carbon number quinolines (C_2, C_4, etc.) which have not been previously reported. Identification is facilitated by the linear relationship between alkyl chain length and retention time. This was particularly important when searching for the low abundance homologues. As both even and odd carbon number HAQs follow the same linear relationship, it is unlikely that the even alkyl HAQs (C_2–C_{12}) represent ring methylated versions of the odd alkyl HAQs. Two sets of unsaturated HAQs were also observed, with one set eluting immediately before the parent quinoline, and the other eluting well afterwards. These may represent either positional or, more likely, geometric isomers.

There is little known about the relative biological activity of the various HAQs and their N-oxides. It is possible that binding to bacterial membranes (and antibacterial activity) will alter with alkyl chain length. Indeed, there is some evidence that the C_5-HAQ is more active against *S. aureus* than the C_7 analogue [7], although both compounds are only weakly active against this organism. The C_7-HAQ N-oxide is far more active against *S. aureus* and other Gram-positive organisms, however, it could account for all of the anti-staphylococcal activity of a pseudomonas extract [7]. In other studies it has been shown that the inhibitory activity of C_9-HAQ N-oxide on respiration by submitochondrial particles from beef heart was 2 or 3 times better than that of the C_7-HAQ N-oxide [15]. Clearly, the biological load (and possible clinical effects) of alkyl-HAQs will depend on the concentration of the various analogues produced by strains of *P. aeruginosa* obtained from patients colonised with the organism. The development of a rapid and simple profiling method based on GC–ECMS will facilitate studies on the biological role of the alkyl-HAQs in patients with chronic pseudomonas infections.

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