Gas chromatographic–mass spectrometric determination of chlorinated cis-1,2-dihydroxycyclohexadienes and chlorocatechols as their boronates

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Abstract

The aerobic degradation of mono- and dichlorobenzenes proceeds via the chlorinated cis-1,2-dihydroxycyclohexa-3,5-dienes, which are subsequently reduced to their chlorocatechols. It is necessary to derivatize the vicinal hydroxyl groups in both polar metabolites to obtain volatile compounds amenable to capillary gas chromatography–mass spectrometry. Butyl- and phenylboronic acids were found to be the most suitable and easy to handle reagents because of their inherent selectivity in forming cyclic boronates. These derivatives were found to be stable and exhibit good chromatographic properties and clear mass spectra, which are presented for all mono- and dichlorocatechols, as well as for 3-chloro-, 3,5- and 3,6-dichloro-cis-1,2-dihydroxycyclohexa-3,5-diene.

1. Introduction

Aerobic microbial degradation of lower chlorinated benzenes starts with the initial oxygenation of the benzene nucleus by (chloro-)-benzenedioxygenases and leads to the chlorinated cis-1,2-dihydroxycyclohexa-3,5-dienes (cis-Ds) [1–3]. These metabolites are substrates for the following dihydroxydihydrobenzene-oxidoreductases, which rearomatize the cis-Ds by dehydrogenation to their chlorinated catechols [1–6]. Subsequently these substituted catechols are transformed by the ring cleaving catechol dioxygenases, with fission mainly occurring between the hydroxyl groups (modified ortho pathway) to the corresponding chlorinated muconic acids. The reaction with a catechol-2,3 dioxygenase

(meta pathway) leads to chlorinated 2-hydroxymuconic semialdehydes, which were reported to be dead-end metabolites that bind irreversibly to enzymes [7].

The degradation of chlorobenzenes by suitable microbes in bioreactors has been recently reported [8]. The efficiency of such biological treatments is controlled by the determination of the chlorobenzene concentrations in inlet and effluent of the bioreactors, as well as by measuring the rate of chloride release and the growth of bacterial populations. Additionally, the screening for exuded metabolites in the effluent is a useful tool for the optimized operation of the reactors with regard to chlorobenzene loading, to temperature and to pH, as well as with regard to oxygen and supplementary substrate concentration. The optimized operation of bioreactors will be described elsewhere. In accordance with

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the observations of other working groups [1,3,5,6], we sometimes observed blue–purple discoloured media during the enrichment of chlorobenzene degrading cultures grown in a chemostat. This phenomenon has been explained as the secretion of chlorinated catechols, which give coloured complexes with transient metal ions and tend to polymerize in their reactive quinone form to yield dark coloured polymerisates. The chlorocatechols are toxic compounds and their secretion might occur at overdoses of substrate [1] or with insufficient availability of catechol dioxygenase.

Knuutinen and Korhonen [9] presented the mass spectral data of all nine underivatized chlorinated catechols and the mass spectra of some derivatized chlorocatechols have also been briefly discussed [10,11]. Chlorinated catechols have been mainly analyzed as their ethyl [10,11], silyl [2,3,12] and acetyl [13] derivatives. These methods exhibit no selectivity for vicinal diols and may result in loaded chromatograms with poor separation of the isomers. Better results were expected by using reagents for the selective derivatization of bifunctional compounds.

As early as 1958, Sugihara and Bowman [14] studied the applications of "benzeneboronic acid" to react with polyhydroxyl compounds to yield stable five-, six- and seven-membered rings. They used the different structures and accordingly different melting and boiling points of cis- and trans-boronate isomers to achieve separation. Brown and Zweifel [15] applied butylboronate esters to separate cis- and trans-cycloalkanediols by distillation. In 1967 Brooks and Watson [16] introduced the use of cyclic boronates into analytical GC–MS and reported that the reaction is specific for certain cyclic cis-diols and can therefore be used to distinguish between cis- and trans-isomers. Brooks and Maclean [17] investigated in 1971 the GC and MS properties of cyclic n-butylboronates as derivatives of polar bifunctional compounds. Later, Brooks and co-workers [18–20] introduced ferroceneboronates as derivatives of diols and related compounds for similar analytical purposes. Poole and Zlatkis [21] reviewed the properties and wide applications of boronic acids to yield cyclic boronic esters. Recently, the use of phenylboronic acid for the

exclusive derivatization of side-chain diols was reported for the chromatography of ecdysteroids [22,23]. To our knowledge, however, the method was not applied for the detection of the first two metabolites of aerobic degradation of chlorobenzenes. The suitability of boronates to analyze catechols selectively with GC–MS was demonstrated with a number of available test substances [24].

In this paper, the GC and MS data of several chlorocatechols and their precursor metabolites, the chlorinated benzene-cis-dihydrodiols as their boronic acid derivatives are reported, as well as their detection in biological samples.

2. Experimental

2.1. Materials

3-Chlorocatechol was kindly provided by F. Lingens, University of Stuttgart-Hohenheim, Germany. 4-Chlorocatechol was purchased from Aldrich, Milwaukee, WI, USA. All four isomeric dichlorocatechols were prepared and stored as previously described [25]. Each catecholic compound was dissolved in acetone to prepare stock solutions containing 1 mg/ml. From these stock solutions standard mixtures containing 1 mg per compound in 10 ml of acetone were prepared.

Butyl- and phenylboronic acids were purchased from Fluka, Buchs, Switzerland. Solutions were prepared by dissolving 20 mg of each boronic acid in 10 ml of dry acetone. All solutions were stored at 4°C in the dark until used.

Acetone, hexane and dichloromethane were Pestanal grade from Riedel-de Haen, Seelze, Germany. Methanol and water for HPLC were Chromosolv grade and purchased from Riedel-de Haen as well.

Analytical-reagent grade sodium sulfate was obtained from Merck, Darmstadt, Germany and dried at 400°C for at least 1 h before use. All other chemicals were of analytical-reagent grade.

2.2. Instrumentation and methods

GC–MS was performed by using a Hewlett-Packard (HP) 5890 Series II gas chromatograph

with hot splitless injector (210°C) and an OV-17 column, 50 m × 0.32 mm I.D. and 0.25 μm film thickness from Seekamp, Achim, Germany. The analytical column was connected to a deactivated, uncoated retention gap of 2 m × 0.32 mm I.D. by a press-fit connector. The injection volume was 2 μl; injection was performed by an automatic liquid sampler HP 7673.

Helium was used as carrier gas with a flow of 3 ml/min. The applied temperature programs differed for butyl- and phenylboronic derivatives, according to optimized separation of the chlorocatechol isomers. The temperature program for butylboronates was: 90°C (2 min), 5°C/min to 140°C (1 min), 10°C/min to 240°C (10 min).

The temperature program for phenylboronates was: 90°C (2 min), 20°C/min to 160°C (10 min), 10°C/min to 240°C (10 min).

The quadrupole mass spectrometer HP 5989 A was operated in the electron impact (EI) mode at 70 eV with an ion source temperature of 240°C and full scan in the range of 50–350 u (1.7 scans/s). When running in the chemical ionization (CI) mode, methane was used as reactant gas with a source pressure of 1.2 Torr (1 Torr = 133.322 Pa) and a source temperature of 170°C.

Preparative HPLC was performed using a Beckman System Gold system equipped with solvent module 126 and UV–Vis detector 166 and a column Beckman Ultrasphere Octyl 5 μm (25 cm × 10 mm). Solvents were methanol (A) and 0.005 M KH$_2$PO$_4$ in water of pH 2.5 (B); the elution was isocratic 30:70 (A–B) with a flow of 4 ml/min. Catechols and dienes were detected at 250 nm.

^{1}H NMR data were obtained from a Bruker AM 400 (Rheinstetten, Germany) instrument, processed with Aspect 3000 processor. NMR was operated with 400 MHz and tetramethylsilane (TMS) as internal standard and C^{2}HCl$_3$ as solvent.

2.3. Extraction of metabolites

A 200–500-ml volume of chemostat medium or bioreactor effluent was centrifuged at 9000 rpm (ca. $1.3 \cdot 10^4$ g) for 20 min. The almost clear supernatant was adjusted to a pH of 10–11 by the addition of 1 M sodium hydroxide solution.

Subsequently, liquid–liquid extraction with 50 ml of n-hexane was performed twice, in order to eliminate all non-polar compounds from the aqueous phase, which then was acidified to pH 4–5 by 1 M hydrochloric acid and extracted twice with 50 ml of dichloromethane. The combined dichloromethane extracts were dried with sodium sulfate and after filtration evaporated to dryness.

2.4. Derivatization procedure

The residue was dissolved in 800 μl of dry acetone, transferred to an autosampler vial and mixed with 200 μl of the boronic acid solution. The vials were capped, shaken and kept at 50°C for 10 min. The extracts so treated were ready for GC–MS analysis. For the preparation of reference chromatograms and spectra, standard solutions or mixtures containing 20 μg per compound in 800 μl of dry acetone were derivatized.

3. Results and discussion

3.1. GC and MS data of monochloro- (MCC) and dichlorocatechol (DCC) isomers after derivatization to boronates

The well known derivatization with boronic acids represents a reversible reaction with an equilibrium constant sufficiently high for the formation of boronate esters in the particular case of coplanar and vicinal diols. A test for long time stability of derivatized extracts was carried out with different vials containing equal amounts of chlorocatechols that were derivatized successively for six days and stored at 4°C until measured on the last day in one sequence. No significant difference between peak areas could be observed with all the derivatives produced within this period of time.

The derivatization of all chlorocatechols used in this study was found to be reproducible and probably complete with both boronic acids, because the plots of the measured areas of the derivative peaks versus concentrations of chlorocatechols resulted in a linear relationship over a wide concentration range. An excess of boronic
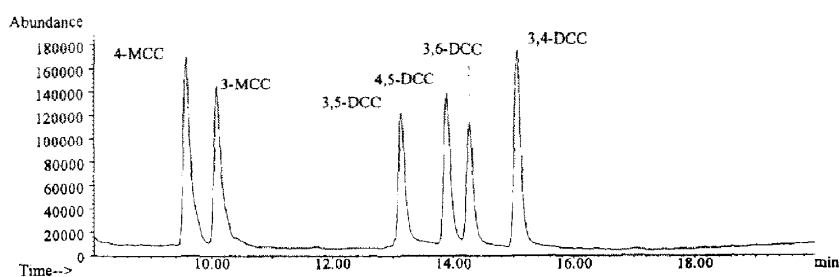
Fig. 1. TIC chromatogram of a standard mixture of all isomeric MCCs and DCCs as their butylboronates.

acid in the reaction mixture was found not to impair the chromatography, because no additional peaks appeared in the relevant part of the chromatogram and the peaks of the derivatives showed good peak shapes (Figs. 1 and 4). The excessive boronic acids were found to react to volatile trimers as described in the literature [21], which was proved by their mass spectra in this study (data not shown).

3.2. Mass spectra of butylboronates

In Fig. 1 a chromatogram of a standard mixture of all MCCs and DCCs after derivatization to their butylboronates is presented. With the GC conditions elaborated in preliminary experiments, a satisfactory separation of all the target compounds can be easily achieved, which is necessary because the isomers show very similar

mass spectra. In Fig. 2, mass spectra of the butylboronates of 3-MCC and 3,6-DCC are presented as examples. The isotopic patterns of boron (^{10}B:^{11}B = 1:4) and chlorine (^{35}Cl:^{37}Cl = 3:1) make the identification of the diol derivatives an easy task.

The spectra of the butylboronates of all MCCs and DCCs exhibit the molecular ion at m/z 210 and 244, respectively, with relative intensities of 30–40%. The base peaks in all spectra are m/z 154 or 188, respectively, which arise from the loss of butene $[M - C_4H_8]^+$. Due to the stability of the cyclic boronate and the favourable loss of butene, all other fragments are of low intensity and negligible.

When negative CI with methane is performed, different but also simple spectra of the butylboronates of MCCs and DCCs are obtained, as can be seen from Fig. 3. Each spectrum provides the molecular ion M^- as base
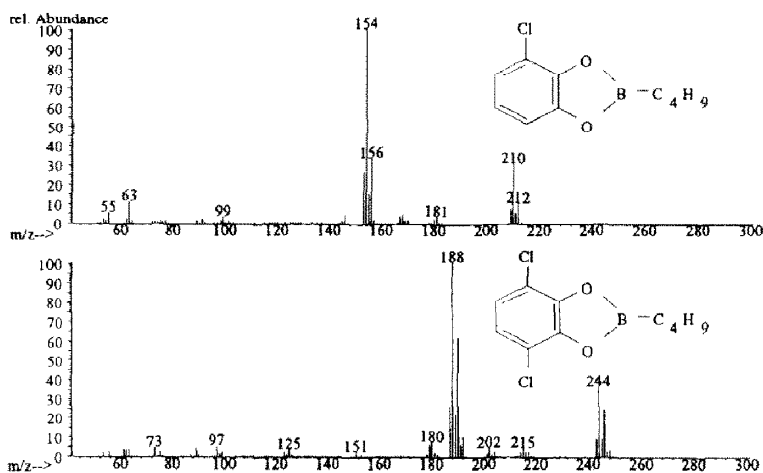

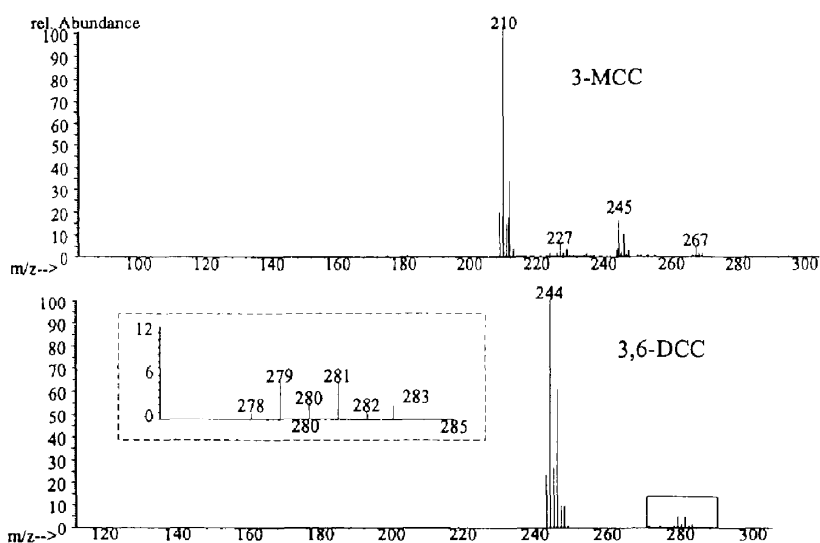Fig. 2. EI mass spectra of 3-MCC and 3,6-DCC as their butylboronates.

Fig. 3. Mass spectra of 3-MCC and 3,6-DCC as their butylboronates obtained with negative CI using methane as reactant gas.

peak without further fragmentation to be observed. Additionally the attachment of a chlorine results in the formation of [M + 35]⁻, which can be easily recognized by checking for the typical chlorine clusters at m/z 245 and m/z 279, respectively.

3.3. Mass spectra of phenylboronates

The total ion current (TIC) chromatogram of a standard mixture of chlorocatechols derivatized with phenylboronic acid is shown in Fig. 4. The chromatogram shows prolonged retention times due to their higher molecular masses with also satisfactory separation of all isomers. Note the different order of elution between 3,6-DCC and 4,5-DCC when compared with butylboronates.

By means of the spectra, the isomers can hardly be distinguished. The great stability of the

Fig. 4. TIC chromatogram of all isomeric MCCs and DCCs as phenylboronates.

phenylboronates shows almost only molecular ions without remarkable fragmentation, as can be seen from the spectra of Fig. 5 for 3-MCC and 3,6-DCC.

Note the ion cluster at m/z 132 with 3,6-DCC, which is obviously the double charged molecular ion M^{2+}, often observed with compounds producing very stable ions. The fragment m/z 123 can be found in any chlorocatechol spectrum in various intensities.

The clusters of the ion m/z 123 indicate that this fragment contains the boron and one chlorine atom. Due to the absence of this fragment ion with the butylboronates, its formation is proposed to occur by loss of the stable boron-phenyl group and the attachment of a cleaved chlorine from the chlorinated catechol to the electrophilic boron.

In Table 1 the intensities of m/z 123 related to M^+ observed with the different derivatives are compiled. They are in agreement with our interpretation of a rearrangement taking place, because the intensities of m/z 123 with the 3-substituted isomers are obviously larger than that observed with other isomers not containing chlorine adjacent to the boron.

The mass spectra obtained from negative CI show the molecular ions M^- without any fragmentation, but in analogy to the butylboronates,
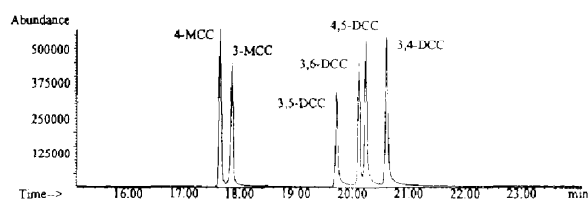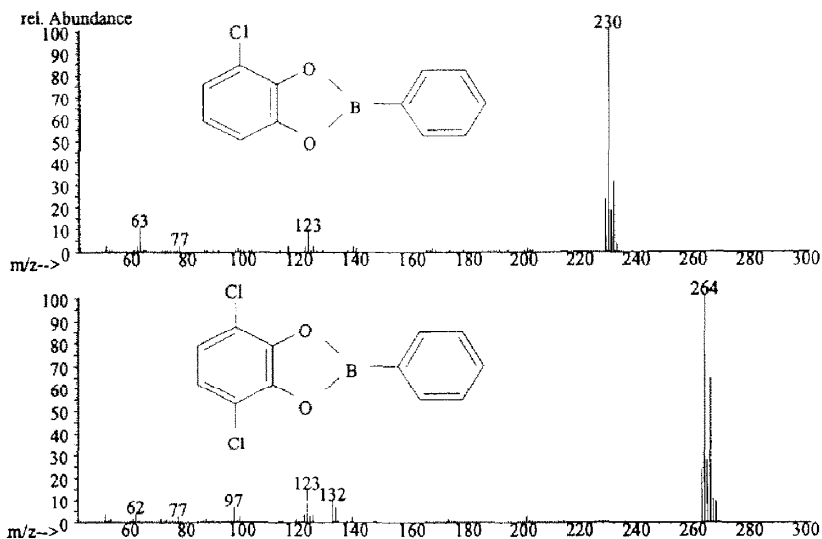
Fig. 5. EI mass spectra of 3-MCC and 3,6-DCC as their phenylboronates.

the attachment of chlorine to [M + 35]⁻ is observed. While the ion m/z 265 in the spectra from 3- and 4-MCC is present with 10–15%, the corresponding ion m/z 299 from DCC spectra is only observed in low abundancies of about 5% of the base peak (data not shown).

3.4. Derivatization of extracts

The derivatization of extracts could be easily carried out without interference by the matrix. Some case studies will be given here as examples. When extracts from bioreactor effluent or

Table 1
Relative intensity of the fragment at m/z 123 from different CCs as their phenylboronates

proposed m/z 123

Chlorinated catechol	Intensity related to M⁺ (%)
3-MCC	9.5
4-MCC	1.7
3,4-DCC	14
3,5-DCC	11
3,6-DCC	15
4,5-DCC	2.7

from medium of the chemostat were analyzed, a standard mixture of chlorocatechols was derivatized and measured within the same sequence, in order to calibrate the system.
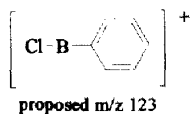
In the first case chosen, the medium of a chemostat was extracted and derivatized with butylboronic acid. This chemostat contained a pure culture of *Pseudomonas aeruginosa* sp., which is able to utilize 1,4-dichlorobenzene (1,4-DCB) only. A typical TIC chromatogram and the relevant spectra of the boronate peaks obtained from such an extract is given in Fig. 6. As can be seen, only small amounts of the expected 3,6-DCC were detectable (10–15 μg/l), whereas another compound appeared at 15.3 min in a larger quantity. The mass spectrum of this compound was not that of a catechol derivative. The spectrum of this unknown compound exhibits ions with isotopic clusters indicative for boron esters (m/z 246, 211 and 146) and such that are indicative for a dichlorinated compound (m/z 246) as well as an one chlorine-containing fragment (m/z 211). Therefore, it is probable a dichloro compound as its butylboronate. The ion with the largest mass is most likely the molecular ion with a mass 2 u higher than that of a DCC butylboronate.

Since the compound shows more fragmentation than the aromatic catechol, a non-aromatic •
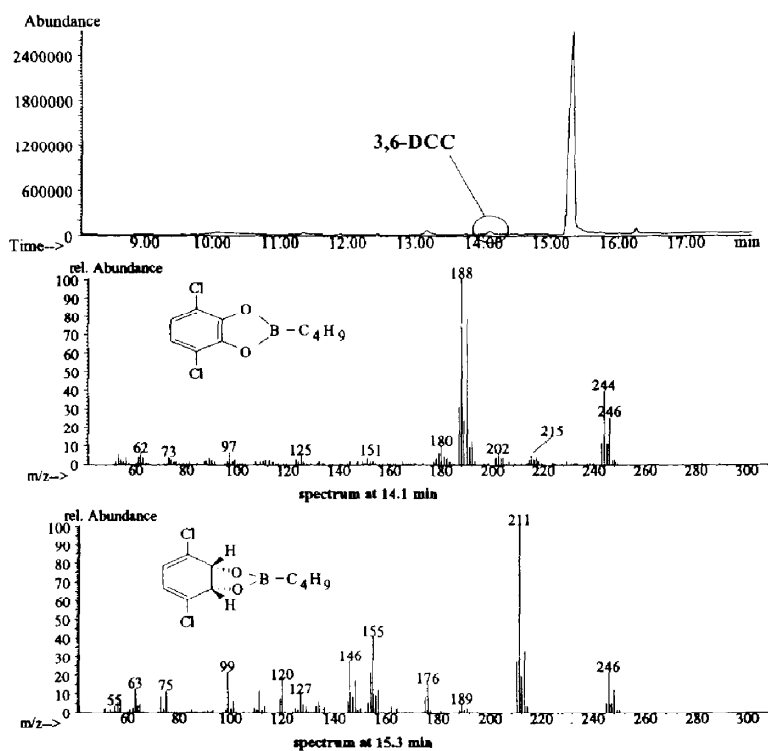
Fig. 6. TIC chromatogram and mass spectra from butylboronates of metabolites extracted from a chemostat experiment eluting at 14.1 and 15.3 min.

structure is probable. The pattern of the chlorine and the boron isotopes at the molecular ion proves the presence of two chlorines in this derivative. The base peak of the spectrum at m/z 211 obviously results from the loss of one chlorine $[M - 35]^+$. The loss of the second chlorine is also observed resulting in m/z 176. Although not all fragments can be reasonably explained, we suggested the presence of the first metabolite of the aerobic degradation of chlorobenzenes, a chlorinated benzene-cis-dihydrodiol. Since the microbes were fed with 1,4-DCB only, this metabolite is strongly indicated to be 3,6-dichloro-cis-D. The structure of the derivatized metabolite is shown in the spectrum of Fig. 6. The content of the cis-diol in the medium of the chemostat was estimated to be 200 μg/l by semi-quantitative analysis. In order to isolate this compound for further characterization, 18 l of chemostat medium were extracted with dichloromethane and the residue was purified by pre-

parative HPLC. The fraction containing the metabolite yielded an amount of 1.9 mg and was subjected to ^{1}H NMR. The spectrum provided two resonances, which were in accordance with the predictions for this symmetrical compound. Related to TMS as internal standard, one singlet was observed at $\delta = 4.45$ ppm, which was assigned to the olefinic protons. A second singlet appeared at $\delta = 6.08$ ppm, assigned to the cis-located protons beneath the hydroxy groups. These data are in agreement with the ^{1}H NMR data of 4-chloro-2,3-dihydroxy-1-methylcyclohexa-4,6-diene, the corresponding metabolite originating from 4-chlorotoluene, reported by Gibson et al. [26].

The isolated cis-dihydrodiol was also measured with negative CI under the conditions described above after derivatization with butylboronic acid. The spectrum is presented in Fig. 7 and shows the quasi molecular ion $[M - 1]^-$ at m/z 245 with an intensity of only 23%. The consider-
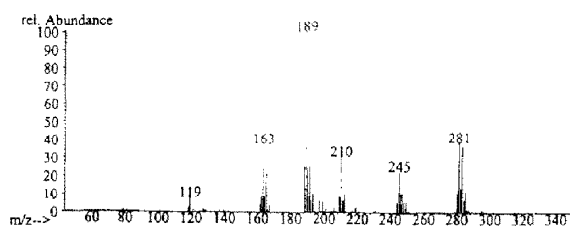
Fig. 7. Mass spectrum of 3,6-dichloro-cis-D obtained with negative CI with methane as reactant gas.

able fragmentation as well as the abstraction of one proton is again indicative to a non-aromatic structure. While m/z 210 originates from the loss of one chlorine, m/z 281 is indicative to an attachment of a chlorine; a reaction that could be observed under the same conditions with butylboronates of DCCs only with intensities below 5% (see Fig. 3). The base peak at m/z 189 is easily explained by the loss of butene.

In a second case study, a bioreactor loaded

with mono- and all three isomers of dichlorobenzene was investigated. The clearly arranged TIC chromatogram of the butylboronates is presented in Fig. 8 and offers only a few peaks. Four of them are marked and the appropriate spectra of compounds I and II are also shown in Fig. 8.

The spectra of compounds I and III can easily be assigned to the structure of MCC and DCC isomers, their retention times were identical with those of 3-MCC and 3,6-DCC. By performing reconstructed ion chromatograms based on m/z 154 and 188, respectively, the presence of other isomers can be checked even in small amounts, but this type of screening was negative in this case.

The spectra of compounds II and IV offer molecular ions, whose masses are again 2 u higher than those of MCC and DCC isomers. The spectra also provide more fragmentation compared to the aromatic catechols. While com-
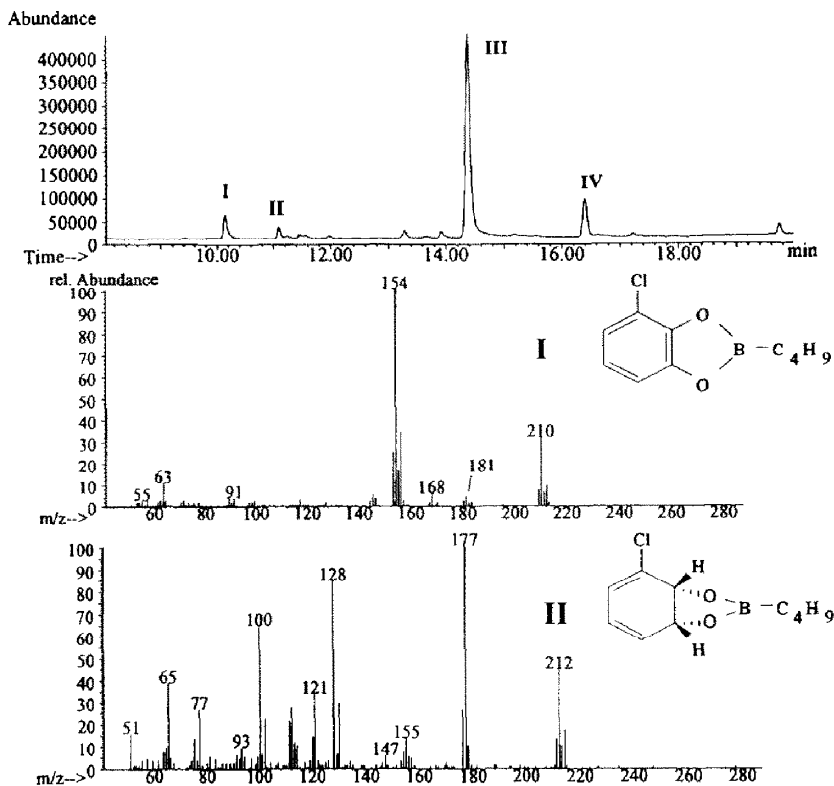

Fig. 8. TIC chromatogram of an extract from bioreactor effluent derivatized with butylboronic acid and mass spectra of two of the compounds indicated.

pound IV shows a spectrum identical to that of 3,6-dichloro-*cis*-D, compound II provides a spectrum unknown so far (see Fig. 8). The boron and chlorine isotope cluster of the probable molecular ion at m/z 212 indicates the presence of one chlorine, whose loss produces the base peak at m/z 177. The fragmentation of M^+ with loss of butene leads to m/z 155, while the cleavage of $O-B-C_4H_9$ leads to the intense peak at m/z 128. Ion m/z 121 can be explained by the loss of butene (C_4H_8) from the fragment ion m/z 177. The appearance of a chlorinated fragment at m/z 100 can be explained by the rearrangement of a chlorinated cyclopentadiene radical.

Summarizing the MS information it is strongly evident that compound II is a chloro-*cis*-D, which probably is the 3-chloro isomer, the natural precursor metabolite of the 3-MCC (compound I in Fig. 8) also detected. The position of the chlorine in 3-MCC is certain (known retention time). Hitherto, the formation of 4-MCC during the degradation of monochlorobenzene could not be observed in our analyses and has not yet been reported by others. Therefore, compound II is unlikely to be 4-chloro-*cis*-D, which would be the natural precursor of 4-MCC.

As a last example, the extract of the medium of a bioreactor was analyzed, loaded with an inlet concentration of about 30 mg/l of 1,3-dichlorobenzene as single substrate. The chromatogram of the butylboronates is presented in Fig. 9, as well as the mass spectra of the dominant peak V at 13.4 min.

The spectrum of compound V eluting at 13.4 min exhibits the ion masses of m/z 246, 211, 189, 146 and 99 already known from the 3,6-dichloro-*cis*-D, so that m/z 246 is probably the molecular ion of this compound (cf. Fig. 6). However, the ion intensities are different and additionally the ions of m/z 162 representing the base peak of the spectrum and m/z 134 next in intensity are found only in this compound. As can be seen, the fragment ion m/z 162 lacks the boron cluster, but contains the double chlorine cluster. Therefore its occurrence can be explained with the cleavage of the $O-B-C_4H_9$ group from the molecular ion with formation of a dichlorophenol ion. Phenols are known to lose CO forming a cyclopentadiene fragment, which is observed with a high abundance at m/z 134 as the corresponding dichloro compound. The cleavage of one chlorine from m/z 134 results in the formation of ion m/z 99, exhibiting the ion cluster of an one chlorine containing fragment. When compared with the 3,6-dichloro-*cis*-D derivative the loss of chlorine seems less favourable and can be
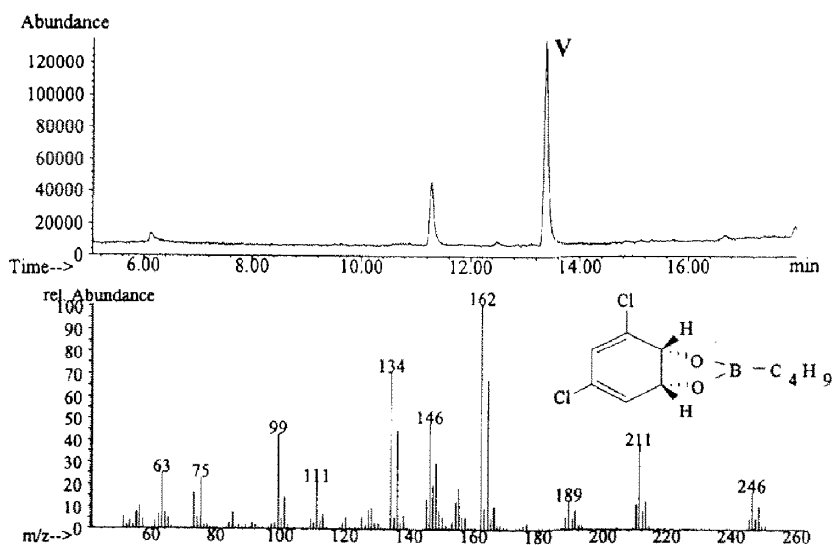

Fig. 9. TIC chromatogram of an extract of a bioreactor effluent dervatized with butylboronic acid and mass spectrum of compound V.

explained only with the different chlorine substitution. Since the microbes of the bioreactor have only degraded the 1,3-dichlorobenzene, it seems reasonable to propose the metabolite obtained to be 3,5-dichloro-cis-D. The structure of its butylboronate is given in Fig. 9. Due to the small amounts produced in the bioreactor it was not possible to isolate this compound in order to support the proposed structure by NMR.

4. Conclusions

For the GC determination of metabolites of the aerobic degradation of chlorobenzenes the derivatization of these compounds is a prerequisite. Butyl- and phenylboronic acids have proven to be suitable reagents for the selective derivatization of diol-containing metabolites. The advantages of using these reagents are: easy handling of the derivatization procedure, quantitative reaction under mild conditions, stability of the derivatives, good GC behavior and clear MS fragmentation pattern. The boron isotope cluster adds to the assignment of fragments. Additionally to note is that boronic acids are less toxic than other derivatization reagents.

The isomers of derivatized chlorocatechols show a good separation with an OV-17 column. The MS detection provides spectra with minor fragmentation and characteristic base peaks for the homologues, whereas the isomers produce very similar spectra.

In contrast, the chlorinated cis-Ds, the precursor metabolites of the chlorocatechols, exhibit significant MS fragmentation, which allows their distinction as could be proved with the 3,5- and 3,6-dichloro-cis-D isomers. The isotopic pattern of boron and chlorine again represent valuable tools for the interpretation of the spectra. The loss of chlorine from the non-aromatic cis-Ds is observed at a significant higher rate than from the more stable chlorocatechols. This also applies to electron capture with negative CI which produces with chlorocatechols almost only the molecular anion while the chlorocyclodienediol boronates show considerable fragmentation.

The screening on the exuded metabolites of

chlorobenzene degradation delivered helpful information about the operation of the bioreactors as well as data about the chemical pathways and properties of microbial metabolism.

Acknowledgement

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