Naphthenic Acids:

Synthesis, Characterisation and Factors Influencing Environmental Fate

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This thesis is dedicated to my mother whose unwavering faith and support have been invaluable!

Thank you.
Abstract

The sustained high price of crude oil is increasing the viability of producing oils and tarry bitumens which have high acid contents and which would have been uneconomical to produce previously. The acidity of these oils is due to the presence of a class of compounds known as Naphthenic Acids (NAs) which are thought to arise from the biodegradation of some of the crude oil hydrocarbons. Some oils contain as much as 3% by weight NAs. The acids cause engineering and production difficulties through corrosion of refinery plant and deposition as salts in pipelines. NAs also cause environmental problems because they are known toxicants. For example, the processing of the vast tar sands deposits of Alberta, Canada results in tailings pond waters containing very high concentrations of NAs. The ponds are estimated to exceed a billion m$^3$ by 2025 and are already visible from space. Bioremediation is an attractive option for reducing the toxicity of such NA wastes. However, an understanding of the biodegradation potential of NAs has previously been hindered by the lack of knowledge of the molecular structure of individual NAs and by the lack of appropriate suitable surrogate acids for detailed mechanistic studies. Although it is known that NAs are complex mixtures of alkyl substituted, mainly alicyclic, carboxylic acids fitting the general formula $C_nH_{2n+1}O_2$, where Z denotes the hydrogen deficiency resulting from ring formation, beyond this, comparatively little was known of NA chemistry, prior to the present study.

The current study describes the synthesis of a series of novel monocyclic surrogate NAs containing both alkyl and alkanoate side-chains (viz: butylcyclohexylbutanoic acids, BCHBAs). The branching in the butyl chain was varied in a systematic way from $n$-through sec-, iso- and tert-butyl. The surrogates were synthesised in high purity (>94%) and rigorously characterised at each synthetic stage by various spectroscopic techniques. A toxicity assay in which developing oyster (Crassostrea gigas) embryos were inoculated with the individual monocyclic surrogate NAs demonstrated EC50 (Effective Concentrations for 50% of population) values ranging from 0.11 mg L$^{-1}$ to 0.49 mg L$^{-1}$. The results exhibited a clear quantitative structure-activity relationship (QSAR) between the degree of branching in the alkyl chain and the toxic effect, with the less branched NAs exhibiting the highest toxicity.

A detailed biodegradation study of the monocyclic surrogate NAs was made. Again a clear QSAR was established. In this instance, $n$-BCHBA was the most readily degraded with 97% degradation by day 9, and tert-BCHBA the most resistant with only 2% degradation by day 30, whereas iso-BCHBA and sec-BCHBA were 77% and 53% degraded after 30 days, respectively. The degradation of the monocyclic surrogate NAs proceeded with the production of major metabolites consistent with $\beta$-oxidation of the alkanoic acid side chain. The metabolites were tentatively identified by mass spectrometry as the ethanoic acid analogues of the butanoic acids. These resisted further biodegradation. That biodegradation proceeded via $\beta$-oxidation was confirmed by the synthesis and subsequent biodegradation experiments with iso-butylcyclohexylmethylbutanoic acid, where the
additional methyl branch in the butanoic acid side chain effectively halted biodegradation. The QSAR observed in the toxicity and biodegradation experiments is thought to be related to the hydrophobicity of the individual NA surrogates, which determines their bioavailability. These results with surrogate compounds help to explain previous reports of the biodegradation of natural NAs in which reductions in toxicity during initial biodegradation have been observed. The surrogate NAs synthesised herein were also used to devise a unique derivatisation procedure which then facilitated the analysis of synthetic and natural NAs by liquid chromatography-multistage mass spectrometry (LC-MS²). Analysis of the resulting naphthenic amides (NAds) allowed an improvement in detection of over two orders in magnitude by positive ion electrospray ionisation-mass spectrometry (ESI-MS) compared to negative ion ESI-MS. The improved response allowed multistage LC-ESI-MS² experiments to be carried out and detailed mass spectral fragmentation pathways for individual NAds to be deduced. Analysis of derivatised natural NA mixtures then allowed a more detailed molecular characterisation of individual NAs than has been possible hitherto.

The use of the surrogate NAs synthesised herein has thus improved significantly our understanding of NA chemistry and the influence of NA structure on the potential for NA bioremediation.
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<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{13}\text{C NMR}$</td>
<td>$^{13}$Carbon nuclear magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>$^1\text{H NMR}$</td>
<td>Proton nuclear magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>DEPT</td>
<td>Distortionless enhancement by polarization transfer</td>
</tr>
<tr>
<td>EI</td>
<td>Electron impact</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionisation</td>
</tr>
<tr>
<td>FID</td>
<td>Flame ionisation detector</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas chromatography-mass spectrometry</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared spectroscopy</td>
</tr>
<tr>
<td>$K_{\text{ow}}$</td>
<td>Octane-water partition coefficient</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>NA</td>
<td>Naphthenic acid</td>
</tr>
<tr>
<td>NAd</td>
<td>Naphthenic amide</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>QSAR</td>
<td>Quantitative structure activity relationship</td>
</tr>
<tr>
<td>TMS</td>
<td>Trimethylsilyl</td>
</tr>
<tr>
<td>UCM</td>
<td>Unresolved complex mixture</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
</tbody>
</table>
Authors Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.
This study was financed by the University of Plymouth and the Petroleum Environmental Geochemistry Group, Plymouth UK.
Relevant scientific seminars and conferences were attended at which work was often presented and a paper has been prepared for publication.

Publications:


Oral presentations and conferences attended:


Signed

Date 20/06/05

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Chapter 1 Introduction

Petroleum acid research dates back to 1874 when Hell and Medinger (Hell and Medinger, 1874) postulated the generic molecular formula that is still accepted to be correct for monocyclic petroleum acids. They concluded that since the acids showed none of the properties of unsaturated acids of the same molecular formula, they must be cyclic. Nine years later in 1883, Markownikoff and Oglobin (Markownikoff and Oglobin, 1883) recognised the petroleum acids definitively as carboxylic acids and suggested the name: naphthenic acids. The term naphthenic acid (NAs) is now the accepted IUPAC nomenclature for petroleum acids (IUPAC Compendium of Chemical Terminology, 1997). The global occurrence of NAs, together with their widespread impact through toxicity, their corrosiveness and their commercial uses has made them very topical substances for study. This has been reflected in the publication of several very thorough reviews by Brient et al., (1995), and more recently by Headley et al., (2004), Clemente and Fedorak, (2005) and Quargraine et al., (2005a). Given the extent and recent dates of these reviews, only a brief summary of NA research is provided herein.

1.1 Chemical structure

It is widely accepted that naphthenic acids comprise a complex mixture of monobasic carboxylic acids represented by the general formula \( C_nH_{2n+z}O_2 \), where \( n \) indicates the carbon number and \( Z \) is a negative even integer specifying a homologous series through hydrogen deficiency (e.g. Brient et al., 1995). NAs are predominantly alkyl substituted cycloaliphatic carboxylic acids, with smaller amounts of acyclic aliphatic acids, and minor amounts of aromatic acids (e.g. Lochte and Littmann, 1955; Seifert, 1975; Brient et al., 1995; Headley and McMartin, 2004; Clemente and Fedorak, 2005). Gas chromatography analysis of NA mixtures reveals an unresolved complex mixture (UCM) or 'hump', similar
to weathered mixtures of petroleum hydrocarbons (e.g., Dzidic et al., 1988; Gough and Rowland, 1990).

Some researchers consider the carboxyl group to be attached to the cyclic moiety via a side chain rather than attached directly, such that cyclopentane acetic acid would be the simplest NA (Figure 1.1a; Rogers et al., 2002a; Hughey et al., 2002; Holowenko et al., 2001; Qian et al., 2001; Brient et al., 1995), whilst others report the carboxylic group to be attached directly to the cyclic moiety, such that cyclopentane carboxylic acid would be the simplest NA (Figure 1.1b; Hsu et al., 2000; Fan, 1991; Dzidic et al., 1988). Owing to the complexity of naphthenic acid mixtures, and current analytical capabilities, it is difficult for analysts to determine whether any additional methylenic carbons are present as alkanolic (e.g., m) or alkyl (e.g., R) moieties, or both (Figure 1.2).

![Figure 1.1. a) Cyclopentane acetic acid b) cyclopentane carboxylic acid.](image)

![Figure 1.2. Generalised structure of a monocyclic naphthenic acid. The cyclic moiety is shown as cyclopentane, but cyclohexane is equally plausible.](image)

It is considered that naphthenic acids containing 7 to 12 carbon atoms comprise monocyclic acids, whilst higher carbon number naphthenic acids comprise multicyclic compounds (Brient et al., 1995). The multiplicity of rings in the structure is demonstrated by the z value where z = 0 for saturated acyclic acids, - 2 for monocyclic acids, and - 4 for bicyclic acids, and so on (examples are shown in Table 1.1). Acyclic naphthenic acids (z = 0) are thought to be branched rather than linear natural fatty acids, and to comprise only a minor component of most NA mixtures (Holowenko et al., 2002). NA size distributions depend on their source, with commercial mixtures which are distilled from crude NAs,
typically ranging in carbon values from 7 to 17, whereas environmental mixtures tend to comprise a broader range. For example, NAs from tailings water in Canada ranged from 7 to 28 carbon atoms (e.g., Scott et al., 2005). The recent discovery of a further group of petroleum acids (i.e. NAs) which fall outside the accepted definition boundaries by having four carboxylic acid groups and an average of 80 carbon atoms (Baugh et al., 2005) is discussed further in Section 1.6.2.

Table 1.1. Typical structures of naphthenic acids, where \( R = \text{alkyl} \). For the acyclic case \( z = 0 \) and the structure is simply \( R-\text{COOH} \). It is possible for \( m \) to equal 0 as explained in the text (adapted from Brient et al., 1995).

<table>
<thead>
<tr>
<th>Monocyclic ( z = -2 )</th>
<th>Bicyclic ( z = -4 )</th>
<th>Tricyclic ( z = -6 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( R-\text{CH}_2\text{CH}_2\text{COOH} )</td>
<td>( R-\text{CH}_2\text{CH}_2\text{COOH} )</td>
<td>( R-\text{CH}_2\text{CH}_2\text{COOH} )</td>
</tr>
<tr>
<td>( R-\text{CH}_2\text{CH}_2\text{COOH} )</td>
<td>( R-\text{CH}_2\text{CH}_2\text{COOH} )</td>
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<td>( R-\text{CH}_2\text{CH}_2\text{COOH} )</td>
<td>( R-\text{CH}_2\text{CH}_2\text{COOH} )</td>
<td>( R-\text{CH}_2\text{CH}_2\text{COOH} )</td>
</tr>
</tbody>
</table>

1.2 Physical and chemical properties

NAs are a family of carboxylic acid surfactants which are viscous liquids, ranging in colour from pale yellow to dark amber. They are completely soluble in organic solvents and oils, but have low solubilities in water (< 50 mg L\(^{-1}\) at pH 7). They have similar acid strengths to higher fatty acids with dissociation constants in the range of \( 10^{-5} \) to \( 10^{-6} \) (Brient et al., 1995). The polarity and non-volatility of NAs increases with molecular weight, giving the individual compounds various physical and chemical properties (Headley and McMartin, 2004) as summarised in Table 1.2.
Table 1.2. Physical and chemical properties of naphthenic acids (Headley and McMartin, 2004).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>General Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colour</td>
<td>Pale yellow, dark amber, yellowish brown, black</td>
</tr>
<tr>
<td>Odour</td>
<td>Primarily imparted by the presence of phenol and sulphur impurities; musty hydrocarbon odour</td>
</tr>
<tr>
<td>State</td>
<td>Viscous liquid</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>Generally between 140 and 450 amu</td>
</tr>
<tr>
<td>Solubility</td>
<td>&lt;50 mg L(^{-1}) at pH 7 in water</td>
</tr>
<tr>
<td>Density</td>
<td>Completely soluble in organic solvents</td>
</tr>
<tr>
<td>Refractive index</td>
<td>Approximately 1.5</td>
</tr>
<tr>
<td>(pK_a)</td>
<td>Between 5 and 6</td>
</tr>
<tr>
<td>(\log K_{ow}) (octanol water partition coefficient)</td>
<td>Approximately 4 at pH 1</td>
</tr>
<tr>
<td>Boiling point</td>
<td>Between 250 and 350 °C</td>
</tr>
</tbody>
</table>

1.3 Occurrence

NAs are common constituents of most crude oils, with crudes from California, Venezuela, Russia and Romania containing as much as 3 % NAs by weight (Brient et al., 1995; Lochte and Littmann, 1955). Perhaps the most significant source of NAs is the Canadian oil sands, the world’s second largest oil reserve, in which NAs are approximately 2 % by weight of the total bitumen samples. Generally, heavy crudes from geologically young formations have the highest acid contents, and paraffinic crudes usually have the lowest. It is believed that some of the NAs present in heavy crude oils are original constituents of the oils, while others result from the chemical and biochemical oxidation of the original oil (Tissot and Welte, 1984). The formation of NAs by biodegradation has been observed in both naturally degraded crude oil (Meredith et al., 2000) and laboratory biodegraded crude oil (Watson et al., 2002).

1.3.1 Canadian oil sands

Canada contains the world’s second largest oil reserves (second only to Saudi Arabia) of 179 million barrels, of which 175 million barrels are located in oil sands (Woynillowicz et al., 2005). The oil sands contain approximately 10-12 % bitumen of which NAs are 2 % by weight. The technique used for extraction of bitumen from the sands is based on the Clarke
caustic wash process. This wash process results in $4 \text{ m}^3$ of fluid tailings for each $\text{m}^3$ of sand processed. These fluid tailings consist of slurry of water, sand, clay and dissolved inorganic and organic compounds, of which NAs are present in concentrations of 20-120 mg L$^{-1}$ (Holowenko et al., 2002). Currently, the crude oil production from the oil sands in Athabasca (Figure 1.3) accounts for more than 25% of Canada’s total production figures, and is expected to rise to over 50% in the next five years (Leung et al., 2003). With a zero discharge policy, the tailings are accumulating to vast volumes with two of the major refineries, Syncrude and Suncor, holding 300 million $\text{m}^3$ up to 1993, and it is estimated that this figure will rise to 1 billion $\text{m}^3$ by 2025 (Quagraine et al., 2005a; Herman et al., 1994). At present, Syncrude’s Mildred Lake site (Fort McMurray, Alberta, Canada) alone contains more than 600 million $\text{m}^3$ of process-affected water (Scott et al., 2005). The extent of the environmental impact of oil sand mining has been brought to light in an independent report published by the Pembina Institute (Alberta Canada) in November 2005. The report, ‘Oil Sands Fever: The environmental implications of Canada’s oil sands rush’, makes recommendations to improve the environmental management of the oil sands and calls for an accelerated transition towards sustainable energy in Canada (Woynillowicz et al., 2005). Photography illustrating the enormity of the environmental impact of the mining and extent of the NA contaminated tailings ponds are shown in Figure 1.4 and Figure 1.5 and some of the stark statistics are summarised below:

- Currently 1/3 of oil produced in Canada comes from oil sands

- To produce one barrel of oil from tar sands:
  
  o 4 tonnes of material is mined
  
  o 2-5 barrels of water are used to extract the bitumen
  
  o Enough natural gas is used in the process to heat 1.5 homes per day

- Target set in 1995 to produce 1 million barrels a day by 2020
- Target reached by 2004
- New target is 5 million barrels per day by 2030

- Emissions in the oil sands industry will double by 2015
- Tailings ponds are some of the largest human-made structures in the world
  - NA concentrations as high as 110 mg L\(^{-1}\)
  - Approx 6 m\(^3\) of tailings produced per 1 m\(^3\) of bitumen produced
  - Syncrude’s Southwest Sand Storage facility is one of three largest dams in the world
  - Existing ponds can be seen from space
  - Syncrude’s Mildred Lake contains more than 400 million m\(^3\) of tailings – enough to fill 160,000 Olympic-sized swimming pools

---

**Figure 1.3.** Map of Canada and Alberta showing the Athabasca oil sands regions (oils sand deposits indicated by dark grey areas on the map of Alberta; Headley and McMartin, 2004).
Figure 1.4. Satellite image of oils sands processing plant, Fort McMurray, Alberta, Canada. The lagoons containing NA-contaminated tailings water are clearly visible. (Source: TerraServer.com).

Figure 1.5. The Suncor upgrader plant and related facilities from the air (Photo: Chris Evans, the Pembina Institute; www.OilSandsWatch.org).
1.4 Surrogate compounds

The complexity of NA mixtures means the identification of individual compounds and investigations of the behaviour of these compounds in toxicity and biodegradation studies has been difficult to date. As a result of the complexity of the 'natural' NAs, surrogate NAs have been used to develop and optimise analytical methods, and to better understand biodegradation pathways. A representative surrogate compound should possess as many of the structural characteristics of the natural compounds as possible. In the case of NAs, the characteristics are an alicyclic (one or more) ring possessing a carboxylic acid group, preferably attached via a methylenic chain, and also an alkyl group. Very few compounds fitting these criteria are commercially available and as a result, studies have been limited to simplistic surrogates often not possessing alkyl substitution or alkanolic chains, and rarely, if ever, both. A detailed list of previously used surrogate NAs can be found in Chapter 2, while a list of the surrogates associated with biodegradation studies can be found in Chapter 3.

1.5 Toxicity

NAs are toxic to a wide variety of organisms including bacteria, zooplankton, plants, fish and rats at aqueous concentrations ranging from 0.1 mg L\(^{-1}\) to over 300 mg L\(^{-1}\) (reviewed in Chapter 3). Their expanding use as wood preservatives owing to their fungicidal properties (Brient et al., 1995) stands testament to their toxic potential. The toxicity of NAs has been attributed to their surfactant character which facilitates their penetration of the cell wall (Quagraine et al., 2005 and references therein). Specifically membrane disruption and cytotoxicity with osmotic stress are suggested to be mainly responsible for the toxic response in molecular studies with \textit{E. coli} (Schramm, 2000). However, again a lack of representative individual surrogate NAs has hampered progress into understanding a relationship between toxicity and NA structure.
The toxicity of oil sand tailings water is well documented (e.g., MacKinnon and Boerger, 1986; Clemente et al., 2004), and it has been suggested that lower molecular weight NAs (n = < 22) contribute most to the toxicity (Holowenko et al., 2002).

1.6 Biodegradation

Biodegradation is the biologically catalysed decomposition of organic matter. It is important not only as a source of NA, via microbial oxidation of petroleum (e.g., Tissot and Welte, 1984), but is also one of the main bioremediation techniques used on NA contaminated sites (Quagraine et al., 2005 a&b).

1.6.1 Formation of naphthenic acids

It has long been established that carboxylic acids are formed during the aerobic biodegradation of petroleum hydrocarbons (Atlas, 1984). For example, one of the formation routes is by the bacterial oxidation of methyl groups to form primary alcohols which are subsequently dehydrogenated via aldehydes to carboxylic acids (Alexander, 1999):

\[
RCH_3 \rightarrow RCH_2OH \rightarrow RCHO \rightarrow RCOOH
\]

Many organic pollutants and natural products contain methyl groups, including alkyl substituted alicyclics which comprise a significant part of the gasoline fraction of petroleum. Numerous studies have demonstrated the oxidation of such alkyl-substituted cyclic hydrocarbons, resulting in cyclohexyl fatty acids with cyclohexane acetic acid as a typical end-point. It is considered that once the terminal methyl group has been readily oxidised, the major pathway of catabolism is β-oxidation (Figure 1.6; Alexander, 1999; Atlas, 1984 and references therein). Aerobic biodegradation of hydrocarbons in subsurface oil has been well documented and has dominated thinking about subsurface biodegradation
for many years (Head et al., 2003). However, recent studies have shown that anaerobic hydrocarbon degradation processes are important on geological time scales, and characteristic metabolites such as decahydro-2-naphthoic acid (a bicyclic NA) have been identified in oils from biodegraded oil reservoirs and in Canadian tar sands (Aitken et al., 2004; Head et al., 2003).

\[ \text{R} \text{OH} \xrightarrow{\beta-oxidation} \text{R} \text{C} \text{O} \text{H} \]

**Figure 1.6.** β-oxidation pathway for the catabolism of alkanoic acids (Alexander, 1999).

### 1.6.2 Biodegradation of naphthenic acids

Aerobic microbial degradation of individual surrogate NAs, commercial mixtures and environmental mixtures of NAs has been demonstrated in numerous laboratory studies (reviewed by Quagraine et al., 2005b). The focus of surrogate NA studies has primarily been to establish biodegradation pathways of the individual acids, whereas studies with commercial and environmental NA mixtures have concentrated on the biodegradation of the compounds as a group (Clemente and Fedorak, 2005). The metabolism of the commercially available surrogate NAs has demonstrated that a wide range of bacteria have the capability to metabolise NAs and that the more recalcitrant structures have the following characteristics: methyl substitution on the cyclic ring of alicyclic acids; β-
substitution of the alkanoic chain; and an even carbon numbered alkanoic substituent on a alicyclic moiety (Quagraine et al., 2005 a&b). A more detailed account of biodegradation of surrogate NAs can be found in Chapter 3.

By far the majority of biodegradation studies involving NA mixtures have focused on the bioremediation of the highly toxic NA contaminated tailing pond waters in Canada which result from tar sand exploration. Microbiologists have shown reductions in toxicity following aerobic biodegradation of both environmental NAs (Mildred Lake Settling Basin, Syncrude; Herman et al., 1994) and commercially available NAs (Kodak salts and Merichem; Clemente et al., 2004) using both laboratory cultures and enrichments of NA-degrading micro organisms. Although a number of researchers have had success with the biodegradation of commercial NA mixtures (e.g., Holowenko et al., 2001; Quagraine et al., 2005a; Scott et al., 2005), studies have not been successful in lowering the concentrations of NAs in tailings water to below 20 mg L\(^{-1}\) (Quargraine et al., 2005). A recent study by Scott et al. (2005) directly compared the biodegradation of four commercial and two oil sand tailings water NA mixtures showing that the commercial mixtures rapidly degraded (>80% reduction in 14 days), while NAs native to the tailings water were a lot more resistant (25 % reduction in 40 days). This was attributed to the presence in the commercial NAs of low molecular mass acids which were readily removed (≤C\(_{17}\)), compared to the more recalcitrant higher molecular masses (≥C\(_{18}\)) of the NAs native to the tailings water. This study was in agreement with a previous study by Watson et al. (2002) where an increase in the concentration of branched and cyclic carboxylic acids with >C\(_{20}\) was observed after extensive biodegradation. These results indicate that biodegradation of commercial NA mixtures does not accurately reflect the bioremediation potential of environmental NAs such as those from the oil sand tailings water, and that real bioremediation potential is held in the degradation of branched cyclic NAs with >C\(_{18}\).
1.7 Economic implications of naphthenic acid

The increasing discovery and development of high total acid number (TAN)-value oil fields from around the world has led to increasing amounts of acidic crudes being processed (Mediaas et al., 2003). There are two economic problems associated with processing these types of crudes; the corrosion of processing equipment and reactions with calcium-ions in the formation water leading to naphthenate deposits blocking process equipment. Largely because of these problems, the high TAN crudes and the Canadian tar sands (Section 1.3.1) were historically uneconomical to produce. However, the increasing price of oil (routinely now above $60 per barrel) has made their production economically viable, therefore there is still a need for corrosion properties and naphthenate deposits to be minimised.

1.7.1 Corrosion

The cost of NA corrosion to the oil industry is significant, through downtime and repair, and it has been claimed that a better understanding of NAs and prevention of corrosion could potentially save several dollars per barrel of oil (Barrow et al., 2003; Turnbull et al., 1998). In 1956, Derungs published an article entitled “Naphthenic Acid Corrosion – An Old Enemy of the Petroleum Industry”. In the article he cites corrosion problems encountered when distilling Romanian, Russian, South American and Californian crudes as early as the 1920s (Derungs, 1956). Of more recent interest is the increasing use of “opportunity” crudes with high NA content, as successful refining of these discounted lower quality crude oils from around the world offers high profitability if refined without major problems (Kane and Cayard, 1999). It has long been recognised that the chief factors effecting NA corrosion are high fluid velocities, operating temperatures between 220 – 400 °C, and the chemical activity of the NAs (Slavcheva et al., 1999; Turnbull et al., 1998; Piehl, 1988: Derungs, 1956). While the nature of the corrosion process depends upon the location of the refinery system, the exact corrosion mechanism has not been conclusively

12
identified (Slavcheva et al., 1999). The corrosive activity of NAs in crude oils has historically been indicated by the total acid number (TAN) of the oil. However, it has increasingly been recognised that the TAN does not always correspond to the corrosiveness of the oil and that knowledge of the individual identities of the NAs being processed would be advantageous for the industry (Barrow et al., 2003; Slavcheva et al., 1999; Turnbull et al., 1998). It is for this reason that spectroscopic methods have been applied to the characterisation of NAs in crude oils to help better understand corrosion (Barrow et al., 2003; Turnbull et al., 1998).

1.7.2 Naphthenate deposition

The hydrophilic nature of NAs means that they congregate at the oil-water interface. As the pressure drops and carbon dioxide is lost from solution during the production, the NAs dissociate to form naphthenate ions. The naphthenate ions then either form stable emulsions as a result of their surfactant characteristics, or react with calcium cations in the aqueous phase to form insoluble naphthenate deposits (Dyer et al., 2003). The naphthenates were first observed in 1996 and were recognised as a threat against continuous production by the end of 1997 (Vindstad et al., 2003). Recent research has shown that the dominating constituents of naphthenate deposits are a family of 4-protic carboxylic acids containing 4 – 8 rings in the hydrocarbon skeleton with molecular weights in the range 1227 – 1235 Da, dubbed the ‘ARN’ acids (Baugh et al., 2005). The identification of the 4-protic ARN acid has not only broadened the views on defining naphthenic acids, but also stands to emphasise that the prediction of corrosiveness and calcium naphthenate deposition by TAN is not accurate.

1.8 Commercial uses

Despite the environmental and refinery problems associated with NAs, they are recovered during oil refinery operations and are sold commercially with more than 5500 t used in
North America in 1992 (Brient et al., 1995). In 1993, 40% of NAs produced were used to make metal salts, primarily copper naphthenate, for use as wood preservatives. NAs are used in a variety of other industrial processes: for improving water resistance and adhesion of concrete; to increase the high pressure resistance of drilling oils; to prevent foaming in jet fuel; in resin manufacture; as flame retardants; to bind steel to rubber in steel radial tyres and in the production of corrosion inhibitors (Clemente and Fedorak, 2005; Brient et al., 1985). Examples of industrial uses of NA metal salts are summarised in Table 1.3 adapted from Brient et al. (1995).

Table 1.3. Examples of industrial uses of naphthenic acids (adapted from Brient et al., 1995).

<table>
<thead>
<tr>
<th>Naphthenic acid metal salt</th>
<th>Industrial use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper naphthenate</td>
<td>Wood preservative</td>
</tr>
<tr>
<td>Zinc naphthenate</td>
<td>Wood preservative and lubricant additive</td>
</tr>
<tr>
<td>Cobalt naphthenate</td>
<td>Tyre production (adhesion promoter) and paint driers</td>
</tr>
<tr>
<td>Manganese naphthenate</td>
<td>Paint drier and fuel additive</td>
</tr>
<tr>
<td>Lead, calcium, iron, cerium and vanadium naphthenates</td>
<td>Paint drier additives</td>
</tr>
<tr>
<td>Sodium and potassium</td>
<td>Emulsifying agents</td>
</tr>
<tr>
<td>Naphthenic amides and imidazolines</td>
<td>Corrosion inhibitors</td>
</tr>
</tbody>
</table>

1.9 Analysis

One of the primary methods for analysis of naphthenic acids is to titrate them with potassium hydroxide to establish the Total Acid Number (TAN). In the commercial supply of naphthenic acids this is often used as the sole indicator of product quality. However, a naphthenic acid TAN only reflects the acidity of the sample. This is a problem particularly encountered in the refinery industry where it has become apparent that the TAN of a crude oil does not necessarily reflect its corrosiveness (Tomczyk et al., 2001; Barrow et al., 2003). The TAN also provides little useful information for chemical, geological or toxicological studies.

A recent review of naphthenic acids (NAs) by Clemente and Fedorak (2005) concluded that “there is currently no method that identifies or quantifies individual acids”. This is
largely due to the complexity of the NA mixture in which hundreds and probably thousands of isomeric compounds are present. For instance, as early as 1969 the late Wolf Seifert and co-workers identified that there were over 1500 acids in a 5% fraction of the carboxylic acids present in a Californian crude oil (Seifert et al., 1969). Thus the application of most analytical separation techniques, such as high performance liquid chromatography (HPLC) or gas chromatography (GC) result in a complex ‘hump’ of unresolved acids, similar to the hump of hydrocarbons present in crude and some refined oils, especially following biodegradation (e.g. Gough and Rowland, 1990). Consequently, NA characterisation has been limited to group identification of n and z numbers, allowing, at best, comparisons of the distributions of such groups in different samples assigned from molecular ions (Figure 1.7).

A wide variety of mass spectrometric techniques have been applied to NAs, with those yielding little or no fragmentation preferred for their identification of molecular ions. MS ionisation methods utilised for the detection of NAs include; electron impact (Seifert et al., 1969; Green et al., 1994; St John et al., 1998; Watson et al., 2002); chemical ionisation (Dzidic et al., 1988; Hsu et al., 2000); fast atom bombardment (Fan, 1991) and electrospray ionisation (Hsu et al., 2000; Headley et al., 2002; Lo et al., 2003). Electrospray ionisation and its applications in NA research are discussed further in Chapter 4. NAs are also routinely analysed by gas chromatography-mass spectrometry following derivatisation to esters (Herman et al., 1994; Green et al, 1994; St John et al., 1998; Jones et al., 2002; Clemente and Fedorak, 2004). NA esters are commonly formed by reacting NA samples with BF$_3$ in methanol or diazomethane (e.g. Jones et al. 2001). However, BF$_3$ in methanol is known to be inefficient in derivatising certain aromatic acids (Watson et al. 2002) and hindered alicyclic acids (Smith, unpublished data).

Hao et al. (2005) used comprehensive two-dimensional gas chromatography/time of flight mass spectrometry (GCxGC/TOF-MS) to analyse commercial and environmental NA
mixtures as methyl esters. They showed that the improvement in chromatographic peak capacity of the GCxGC and the ability of TOF-MS to acquire up to 500 spectra/s combined with the deconvolution software, facilitated the extraction of mass spectra of resolved NA isomers. They believe that prior separation and further instrument optimisation will allow the identification of individual toxic components of NA mixtures. However, interpretation of the electron impact mass spectra may still be the limiting factor in the structural characterisation of individual NA isomers.

Quantification of NAs has been achieved with Fourier transform infrared (FTIR) spectroscopy and more recently high performance liquid chromatography coupled to an ultra violet (UV)-visible diode array detector. The FTIR method measures the monomeric and dimer forms of the carboxylic acid groups following quantitative extraction with dichloromethane from aqueous samples (Holowenko et al., 2001; Yen et al., 2004). The detection limits for this technique are typically in the region of a few tenths of a milligram per litre of aqueous sample extracted. The HPLC method quantifies 2-nitrophenylhydrazine derivatives of NAs. The derivatised acids elute from a C18 column as an unresolved hump which is integrated and quantified by comparison to calibration curves of commercial NAs. Detection limits as low as 5 mg NAs per litre of aqueous sample have been reported (Clemente et al., 2003b; Yen et al., 2004). Electrospray ionisation MS has also been used to quantify NAs in aqueous solutions with detection limits as low as 0.01 mg L\(^{-1}\) (Headley et al., 2002; Lo et al., 2003).
Figure 1.7. Carbon number distribution of ring types (based on $z$ number) in a sample of naphthenic acids extracted from oil sands tailings water. Results obtained from GC–MS analysis of $t$-butyldimethylsilyl derivatives of the acids (Clemente and Fedorak, 2005).
1.10 Present study

The foregoing literature review (Chapter 1) helps to demonstrate that NAs are a widespread, complex mixture of largely unidentified carboxylic acids with proven toxicity to a wide range of organisms, and a potent corrosive potential to refinery plants. A better knowledge of their structural composition on a molecular level is essential, not only in environmental regulation and bioremediation, but also in the development of corrosion inhibitors. It is widely acknowledged that there is currently no method that allows identification or quantification of individual acids. As a consequence many studies have utilised surrogate NAs to both elucidate biodegradation pathways and also to optimise analytical techniques. However, the true representation of the commercially available surrogate compounds is questionable as many do not contain the agreed key characteristics of NAs. This issue is addressed further in Chapter 2, where the synthesis and characterisation of a series of more representative surrogate compounds possessing a cyclic moiety substituted with both alkyl and alkanoic groups is described. The surrogate NAs were then utilised in Chapter 3 for toxicity and biodegradation bioassays. The toxicity tests demonstrated the toxicity of the surrogates, over a range of concentrations, to developing oyster embryos (Crassostrea gigas). Biodegradation of the surrogates by mixed cultures of bacteria with a focus on bioremediation is also described. The results show a quantitative structure-activity relation (QSAR) in which the branching of the alkyl (butyl) substituent influences both the toxic effect to developing embryos, and the rate of biodegradation. Chapter 4 describes the development of a unique derivatisation method developed herein for NAs which allows identification by positive ion electrospray ionisation-mass spectrometry detection. The development of analytical separation using the synthetic and commercial surrogates is also described, followed by analysis of 'real' environmental NA mixtures with molecular level structural identification for the first time. The experimental details for Chapters 2 to 4 are described in Chapter 5. Chapter 6
summarises the conclusions of Chapters 2 to 4 and outlines a number of areas for future work.
Chapter 2 Synthesis of surrogate naphthenic acids

2 Introduction

The formulation and use of surrogate compounds for method development and optimisation is an invaluable tool in characterisation studies of complex mixtures of unknown compounds such as naphthenic acids (NAs; e.g. Dzidic et al., 1988; St John et al., 1998; Holowenko et al., 2001; Rogers et al., 2002a). Once selected, surrogate compounds can be used individually as well as in mixtures to optimise analytical techniques, particularly those involving chromatographic separation and spectroscopic detection. Surrogates have proved to be invaluable in studies aimed at assessing the environmental threat and persistence of NAs, where they have been used to elucidate the pathways of the biodegradation of individual NAs (reviewed by Clemente and Fedorak, 2005). Similarly, surrogates of other compound classes have been used to establish toxicological structure activity relationships (SARs; e.g. Donkin et al., 1989).

Surrogate NAs studied previously (Figure 2.1 a-j) include camphoric acid (a), 1-methyl-1-cyclohexanecarboxylic acid (b), and n-decanoic acid (c; St John et al., 1998); 3-cyclohexylpropanoic acid (d), 4-cyclohexylbutanoic acid (e), 5-cyclohexylpentanoic acid (f), and 6-phenylhexanoic acid (g; Holowenko et al., 2001); 4-methylcyclohexylethanoic acid (h); 4-methylcyclohexanecarboxylic acid (i; Peng et al., 2002) and decahydro-2-naphthoic acid (j; Thomas, 1995; Lai et al., 1996). However, useful though these studies have been in aiding for example, analytical method developments (St John et al., 1998; Clemente et al., 2004) and studies of biodegradation pathways (Herman et al., 1994; Lai et al., 1996; Holowenko et al., 2001, Clemente et al., 2003a) it is questionable how representative of real NAs the surrogates are since most studies have concluded that NAs comprise cyclic compounds possessing both alkyl and alkanoate substituents (Figure 2.2; reviewed by Brient, 1995), whereas those shown in Figure 2.1 (except h) possess only one
or other of these structural features, not both. Thus, there is a need for synthesis of acids more representative of those found in the environment.

![Figure 2.1. Examples of surrogate naphthenic acids previously used (compound names are given in the text).](image)

![Figure 2.2. Generalised structure proposed for a naphthenic acid (the cyclic moiety represented by a cyclopentyl ring may be a cyclohexyl ring or a polycyclic moiety; e.g. Brient et al., 1995; Clemente and Fedorak, 2005).](image)

### 2.1 Aims

The aim of the work described in the present chapter was to synthesise at least milligram quantities of a number of well characterised, pure, alicyclic surrogate NAs containing both alkyl and alkanoate substituents. Such compounds are not commercially available, and hence no previous studies have included these components even though many reviewers have suggested that 'real' NAs contain both features—as shown in Figure 2.2 (e.g. Brient et al., 1995; Clemente and Fedorak, 2005). Thus, a series of 4-(4'-butylcyclohexyl)butanoic acids was synthesised and characterised.
2.2 Synthetic strategy

The chosen surrogates (Figure 2.3), 4-(4'-n-butylcyclohexyl)butanoic acid, 4-(4'-2-methylpropylcyclohexyl)butanoic acid, 4-(4'-1-methylpropylcyclohexyl)butanoic acid and 4-(4'-1,1-dimethylethylcyclohexyl)butanoic acid (abbreviated hereafter as n-BCHBA, iso-BCHBA, sec-BCHBA and tert-BCHBA, respectively; Figure 2.3) were synthesised using a modification of the Haworth synthesis (Figure 2.4; Vogel, 1989; March, 1985). This involved the reaction of an aromatic hydrocarbon with either a lactone or an anhydride, via a Friedel-Crafts acylation reaction to form an aromatic keto acid. The aromatic keto acid was then reduced to an aromatic acid by Clemmensen reduction, the product of which was hydrogenated to give an alicyclic surrogate naphthenic acid. The products of each synthetic pathway were characterised by gas chromatography-mass spectrometry (GC-MS), nuclear magnetic resonance spectroscopy (NMR) and infrared spectroscopy (IR).

\[4-(4'-n\text{-butylcyclohexyl})\text{butanoic acid} \quad (n\text{-BCHBA})\]

\[4-(4'-iso\text{-butylcyclohexyl})\text{butanoic acid} \quad (iso\text{-BCHBA})\]

\[4-(4'-sec\text{-butylcyclohexyl})\text{butanoic acid} \quad (sec\text{-BCHBA})\]

\[4-(4'-tert\text{-butylcyclohexyl})\text{butanoic acid} \quad (tert\text{-BCHBA})\]

**Figure 2.3.** Structures, names and abbreviations of the synthesised surrogate naphthenic acids.
2.3 Synthesis of 4-(4'-iso-butylecyclohexyl)butanoic acid (iso-BCHBA)

Full experimental details for the synthesis of iso-BCHBA can be found in Chapter 5.

2.3.1 Preparation and characterisation of 4-(4'-iso-butylphenyl)-4-oxobutanoic acid

4-(4'-iso-butylphenyl)-4-oxobutanoic acid (Figure 2.5; I) was formed in good yield (59%) via a Friedel-Crafts acylation of iso-butylbenzene using succinic anhydride, with aluminium chloride as a catalyst, and tetrachloroethane as the solvent (Figure 2.5). Figure 2.6 shows a GC-MS total ion current chromatogram of 4-(4'-iso-butylphenyl)-4-oxobutanoic acid as the trimethylsilyl (TMS) ester. Integration of the GC peak areas showed the crude keto acid (I) to be >97% pure with no one impurity > 1%. Therefore, further purification was deemed unnecessary.
Figure 2.5. Reaction scheme for the synthesis of 4-(4'-iso-butylphenyl)-4-oxobutanoic acid (I).

Confirmation of the structure of I was provided by mass spectrometry where the molecular ion (M⁺, m/z 306) was observed in the electron impact mass spectrum of the TMS ester (Figure 2.7). The ease of loss of the methyl group of the TMS ester accounts for the relative low intensity of the molecular ion and for the ion at m/z 291 (M⁺ -CH₃), characteristic of TMS esters. The low intensity ion at m/z 263 results from benzylic cleavage of the iso-butyl side chain, while the base peak at m/z 161 results from benzylic cleavage to leave a 4-iso-butylbenzoyl fragment (Figure 2.7).

Figure 2.6. Total ion current gas chromatogram of 4-(4'-iso-butylphenyl)-4-oxobutanoic acid (as TMS ester). (GC-MS conditions: Column, HP-1MS 30 m x 0.25 mm id x 0.25 μm film; oven programme; 40 – 300 °C at 10 °C min⁻¹, hold 10 min; injector 250 °C).
Figure 2.7. Mass spectrum of 4-(4'-iso-butylphenyl)-4-oxobutanoic acid (as TMS ester). (MS conditions: Ion source 250 °C, ionisation energy 70 eV, mass range 50 -550 Daltons).

Examination of the keto acid (I) by $^1$H, $^{13}$C and DEPT NMR spectroscopy (Figures 2.8 to 2.11) led to complete assignment (Table 2.1). The numbering system used for NMR characterisation was chosen for simplicity and does not correspond to the systematic numbering used in the naming of the compounds. The $^1$H NMR spectrum of 4-(4'-iso-butylphenyl)-4-oxobutanoic acid was characterised by an intense doublet (integral 6.3H) of resonances at 0.89 ppm assigned to the six protons of two methyl groups (H-1). The signal splitting (doublet) was attributed to the effect of an adjacent single neighbouring proton (H-2). This feature is typical of the $^1$H NMR spectra of compounds containing the isopropyl (>CH(CH₃)₂) moiety and thus helps to confirm the presence of the iso-butyl side chain. A multiplet at 1.88 ppm (integral 1H) was attributed to H-2, and the multiplicity was expected from the presence of the six methyl protons (H-1) and two adjacent methylenic protons (H-3). In turn, the influence of the only neighbouring proton H-2 on the splitting of the resonance signal of the methylenic protons (H-3) was consistent with the presence of a doublet (integral 2H) at 2.52ppm assigned to H-3. This effectively establishes the presence of the iso-butyl group substituent (Figure 2.8). The aromatic protons (H-5 and H-6) are
observed as two doublets at 7.22 and 7.88 ppm (integrals of 2.4H and 2.0H, respectively). The two doublets confirm that each aromatic proton has only one neighbouring proton, thus confirming that butanoic keto acid substitution had occurred para to the position of the isobutyl group. The remaining protons were observed as triplets at 2.77 (integral 2H) and 3.24 ppm (integral 2H) and were assigned to H-10 and H-9, respectively. The triplet at 3.24 ppm was assigned to H-9 as it was considered that the interaction with the neighbouring carbonyl carbon would have a greater downfield effect than that of the interaction of H-10 (2.77 ppm) with the carboxyl group.

The $^{13}$C NMR spectrum of 4-(4'-iso-butylphenyl)-4-oxobutanoic acid (Figure 2.10) shows an intense resonance at 22.3 ppm which remains above the baseline in the DEPT spectrum (Figure 2.11) indicating that it is due to either a tertiary or a methyl carbon. The DEPT spectrum shows one other resonance (30.3 ppm) that remains above the baseline in the 0 – 50 ppm region which is approximately half the intensity of that at 22.3 ppm. Thus the resonance at 22.3 ppm is attributed to the two methyl groups of the isopropyl moiety (C-1) and the resonance at 30.3 ppm is attributed to tertiary carbon (C-2) of the same moiety.

The resonances represented by peaks below the baseline in the DEPT spectrum (Figure 2.11) are due to secondary methylenic (-CH$_2$-) carbons (C-3, C-9 and C-10). C-3 is assigned to the resonance at 45.5 ppm as lack of shielding from the aromatic group causes a downfield shift compared to C-9 and C-10. This assignment is further supported by signals observed in the NMR spectrum of iso-butylbenzene (Appendix 1). C-9 is assigned to the resonance at 33.3 ppm while C-10 is assigned to that at 28.0 ppm. The carbonyl (C-8) and carboxyl carbons (C-11) show resonances at 197.9 and 178.3 ppm, respectively and as is characteristic of quaternary carbons, neither are represented by signals on the DEPT spectrum (Williams and Fleming, 1973). The aromatic carbons show resonances between 125 – 150 ppm, of which the resonances at 134.3 and 148.0 ppm are not present on the DEPT spectrum and so are assigned to the substituted quaternary C-4 and C-7. It is likely
that the influence of the carbonyl carbon (C-8) has caused the resonance of C-7 to be at slightly higher field than that of C-4. The four remaining carbons of the aromatic ring are seen as two resonances (128.0 and 129.4 ppm), which provides further evidence that the compound is para disubstituted.

![Chemical Shift (ppm)](image)

**Figure 2.8.** $^1$H NMR spectrum of 4-(4'-iso-butylyphenyl)-4-oxobutanoic acid. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform. Table 2.1 contains details of chemical shifts and integrals.)
Figure 2.9. $^1$H NMR spectrum of 4-(4'-iso-butylphenyl)-4-oxobutanoic acid, expanded region between 0.5 and 3.5 ppm. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform. Table 2.1 contains details of chemical shifts and integrals).

Figure 2.10. $^{13}$C NMR spectrum of 4-(4'-iso-butylphenyl)-4-oxobutanoic acid. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform. Table 2.1 contains details of chemical shifts and integrals).
Figure 2.11. $^{13}$C DEPT NMR spectra of 4-(4'-iso-butylphenyl)-4-oxobutanoic acid. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform. Table 2.1 contains details of chemical shifts and integrals).

**Table 2.1.** Assignment for $^1$H and $^{13}$C resonances in NMR spectra of 4-(4'-iso-butylphenyl)-4-oxobutanoic acid.

<table>
<thead>
<tr>
<th>Carbon number</th>
<th>$^1$H chemical shift (Integral)</th>
<th>$^1$H chemical shift (Hz)</th>
<th>$^{13}$C chemical shift (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CH$_3$</td>
<td>CH$_2$</td>
</tr>
<tr>
<td>1</td>
<td>0.89 (6.3)</td>
<td>5.4</td>
<td>22.3</td>
</tr>
<tr>
<td>2</td>
<td>1.88 (1.0)</td>
<td>5.4</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2.52 (2.0)</td>
<td>8.1</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>7.22 (2.4)</td>
<td>8.1</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>7.88 (2.0)</td>
<td>8.1</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>3.24 (2.0)</td>
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<tr>
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</table>

Infrared spectroscopic examination of 4-(4'-iso-butylphenyl)-4-oxobutanoic acid (Figure 2.12) showed a characteristic broad absorption band at 3423 cm$^{-1}$ due to the hydrogen bonded carboxylic acid –OH group (stretching), and a sharp peak at 3032 cm$^{-1}$ due to the
free carboxylic O-H stretch. Two peaks at 1699 cm⁻¹ and 1678 cm⁻¹ are assigned to carboxyl and carbonyl stretching, respectively. Strong bands at 2956, 2922 and 2870 cm⁻¹ were assigned to >CH₂, –CH₃ and C-H bond stretching. The sharp bands at 1603 and 1586 cm⁻¹ were assigned to aromatic C-H bends, whilst the sharp band at 839 cm⁻¹ is characteristic of para-disubstitution. The small band at 1381 cm⁻¹ is characteristic of an isopropyl (>C(CH₃)₂) moiety, and is further confirmation of the presence of the iso-butyl chain. Other absorptions observed in the fingerprint region were likely to be due to aliphatic C-H bending (~1350 – 1500 cm⁻¹) and the C-O stretch of the carboxylic acid (~1200 – 1300 cm⁻¹).

Figure 2.12. Infrared spectrum of 4-(4'-iso-butylphenyl)-4-oxobutanoic acid (KBr disc).

2.3.2 Preparation and characterisation of 4-(4'-iso-butylphenyl)butanoic acid

4-(4'-iso-butylphenyl)-4-oxobutanoic acid (I) was converted into 4-(4'-iso-butylphenyl)butanoic acid (II) via a Huang-Minlon modification of a Wolff-Kischner reduction (Huang-Minlon, 1946) using hydrazine hydrate with potassium hydroxide and ethylene glycol as solvent (Figure 2.13). Acid (II) was obtained in good yield (84%) and purity (>97%) as shown by GC-MS analysis of the TMS ester (Figure 2.14).
Figure 2.13. Reaction scheme for the synthesis of 4-(4'-iso-butylphenyl)butanoic acid (II).

The mass spectrum of the TMS ester (Figure 2.15) contained a molecular ion at $m/z$ 292, and an ion at $m/z$ 277, which corresponds to $M^+ - 15$ (i.e. the loss of a methyl group). The ion at $m/z$ 160 was the result of $\gamma$-H rearrangement known as the McLafferty rearrangement which occurs in compounds containing an unsaturated functionality (the carbonyl group in this case) along with a $\gamma$-H (McLafferty and Tureček, 1993). Cleavage at the esteric carbon results in an ion at $m/z$ 117 ($^1$COOTMS) which is characteristic of many TMS esters.

Figure 2.14. Total ion current gas chromatogram of 4-(4'-iso-butylphenyl)butanoic acid (as TMS ester). (GC-MS conditions: Column, HP-1MS 30 m x 0.25 mm id x 0.25 μm film; oven programme; 40 – 300 °C at 10 °C min$^{-1}$, hold 10 min; injector 250 °C).
Figure 2.15. Mass spectrum of 4-(4'-iso-butylphenyl)butanoic acid (as TMS ester). (MS conditions: ion source 250 °C, ionisation energy 70 eV, mass range 50-550 Daltons).

Analysis of II by $^1$H, $^{13}$C and DEPT NMR spectroscopy (Figures 2.16 to 2.19, respectively) led to complete assignment of proton and carbon chemical shifts (Table 2.2). Reduction of the keto group had very little effect on the resonances of the protons in the iso-butyl side chain; thus the resonances are assigned as described in the previous section (H-1 0.89 ppm; H-2 1.84 ppm; H-3 2.44 ppm; section 2.4.1). The proton spectrum also shows that the aromatic protons are now magnetically equivalent and thus observed as a singlet (H5 and H6 7.07 ppm; integral 3.8H). A triplet at 2.37 ppm (integral 2H) was assigned to the methylenic protons at H-8 which arise as a result of the reduction of the carbonyl group. As expected, the chemical shift was similar to that of H-3 because both protons are adjacent to the aromatic ring. The multiplet at 1.96 ppm (integral 2H) was attributed to H-9 and the multiplicity was as expected from the presence of four adjacent methylenic protons (H8 and H10). The remaining triplet at 2.61 ppm (integral 2H) was attributed to H-10. The notable differences in the $^{13}$C and DEPT spectra to those of 4-(4'-
iso-butylphenyl)-4-oxobutanoic acid are the loss of the carbonyl resonance at 197.9 ppm and the appearance of a resonance signal at 34.5 ppm (C-8). Otherwise, the relative order of peak shift assignments was as for 4-(4'-iso-butylphenyl)-4-oxobutanoic acid.
Figure 2.16. $^1$H NMR spectrum of 4-(4'-iso-butylphenyl)butanoic acid. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform. Table 2.2 contains details of chemical shifts and integrals).

Figure 2.17. $^1$H NMR spectrum of 4-(4'-iso-butylphenyl)butanoic acid, expanded region between 0.5 and 3.0 ppm. (Joel EX270MHz HR FT-NMR. Samples dissolved in deuterated chloroform. Table 2.2 contains chemical shifts and integrals).
Figure 2.18. $^{13}$C NMR spectrum of 4-(4'-iso-butylphenyl)butanoic acid. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform. Table 2.2 contains chemical shifts).

Figure 2.19. DEPT NMR spectrum of 4-(4'-iso-butylphenyl)butanoic acid. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform, Table 2.2 contains chemical shifts).
Table 2.2. Assignment of $^1$H and $^{13}$C resonances in NMR spectra of 4-(4'-iso-butylyphenyl)butanoic acid.

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<th>J (Hz)</th>
<th>$^{13}$C chemical shift (ppm)</th>
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</tbody>
</table>

1 Protons for 5 and 6 are in identical environments.
2 Assignments of C4 and C7 may be interchanged.
3 Assignments of C5 and C6 may be interchanged.

The infrared spectrum (Figure 2.20) was very similar to that described for 4-(4'-iso-butylyphenyl)-4-oxobutanoic acid in the previous section with the notable difference that the absorbance due to the C=O (1768 cm$^{-1}$) in the keto group was absent in the spectrum of II.
2.3.3 Preparation and characterisation of 4-(4'-iso-butylcyclohexyl)butanoic acid

4-(4'-iso-butylphenyl)butanoic acid (II) was converted into 4-(4'-iso-butylcyclohexyl)butanoic acid (Figure 2.21; III) via a hydrogenation reaction adapted from that of Dauben and Hoerger (1951), using platinum oxide as the catalyst and acetic acid as the solvent with a steady stream of hydrogen under pressure (ca. 8 psi). 4-(4'-iso-butylcyclohexyl)butanoic acid was obtained in quantitative amounts (ca. 200 mg) and high purity (>99%) as two isomers shown by GC-MS analysis of the TMS esters (Figure 2.24).

**Figure 2.20.** Infrared spectrum of 4-(4'-iso-butylphenyl)butanoic acid (KBr disc).

**Figure 2.21.** Reaction scheme for hydrogenation of 4-(4'-iso-butylphenyl)butanoic acid (II) to the surrogate naphthenic acid 4-(4'-iso-butylcyclohexyl)butanoic acid (iso-BCHBA; III).

Mass spectrometry, in conjunction with NMR and IR spectroscopy, confirmed the structure of 4-(4'-iso-butylcyclohexyl)butanoic acid. Hydrogenation of the benzene ring reduced it to a cyclohexyl ring on which the two substituents were fixed in either cis or
trans configurations giving rise to two diastereomers. The trans configuration (Figure 2.22) has substituents in diequatorial and dixial conformations, while the cis configuration (Figure 2.23) has substituents in equatorial-axial and axial-equatorial conformations. The conformers of each configuration were not resolved under GC conditions applied herein. The stability of the different conformations of each configuration gave rise to different ratios of the two diastereomers (Figure 2.24).

![Diequatorial](image1.png) ![Dixial](image2.png)

**Figure 2.22.** Chair conformations of trans-4-(4'-iso-butylcyclohexyl)butanoic acid (R = iso-butyl; R' = butanoic acid).

![Equatorial-axial](image3.png) ![Axial-equatorial](image4.png)

**Figure 2.23.** Chair conformations of cis-4-(4'-iso-butylcyclohexyl)butanoic acid (R = iso-butyl; R' = butanoic acid).

The mass spectra (Figure 2.25) of both isomers were identical, each exhibiting a molecular ion of m/z 298 (as TMS ester) as expected. Both the loss of a methyl (m/z 283; M⁺ - 15) and a base peak ion of m/z 117 are characteristic of TMS esters of carboxylic acids. The ion observed at m/z 255 (M⁺ - 43) could be attributed to the cleavage of the isopropyl moiety. However, this ion was also present in the mass spectra of sec-BCHBA and tert-BCHBA (Figure 2.26 and Figure 2.27, respectively) where such a fragmentation is not possible. The mass spectrum of cyclohexylbutanoic acid (CHBA; Figure 2.28) as a TMS ester exhibited ions at M⁺ - 43 and M⁺ - 59, also present in the mass spectrum for iso-BCHBA (m/z 239 and 255). The presence of these losses in the mass spectrum of CHBA
suggests that these ions were due to a rearrangement where the esteric carbon joined the ring on the adjacent carbon, resulting in the loss of a C3 unit from the butanoic acid alkyl moiety. The mass spectrum of 4-(4'-iso-butylcyclohexyl)pentanoic acid (as TMS ester; Figure 2.29) further supports this as ions are observed at $M^+ - 57$ and $M^+ - 73$. These ions are 14 Da higher than previously discussed, consistent with the methyl branch on the butanoic acid moiety causing a C4 unit to be lost (Figure 2.30). Mass spectra of analogous structures suggest that such losses are unique to carboxylic acid TMS esters with a four carbon backbone attached to a ring structure, and that a rearrangement involving the TMS ester group and the ring had occurred. There appears to be no literature precedent for this.

Figure 2.24. Total ion current gas chromatogram of 4-(4'-iso-butylcyclohexyl)butanoic acid conformers (as TMS esters). Inset: expanded region of chromatogram 15.6 - 16.8 min. (GC-MS conditions: Column, HP-1MS 30 m x 0.25 mm id x 0.25 μm film; oven programme; 40 - 300 °C at 10 °C min$^{-1}$, hold 10 min; injector 250 °C, ion source 280 °C, 70 eV).
Figure 2.25. Representative mass spectrum of 4-(4'-iso-butylocyclohexyl)butanoic acid conformers (as TMS ester). (MS conditions: Ion source 250 °C, ionisation energy 70 eV, mass range 50 -550 Daltons).

Figure 2.26. Mass spectrum of 4-(4'-sec-butylocyclohexyl)butanoic acid (as TMS ester). (MS conditions: Ion source 250 °C, ionisation energy 70 eV, mass range 50 -550 Daltons).
Figure 2.27. Mass spectrum of 4-(4'-tert-butylicyclohexyl)butanoic acid (as TMS ester). (MS conditions: Ion source 250 °C, ionisation energy 70 eV, mass range 50-550 Daltons).

Figure 2.28. Mass spectrum of butylcyclohexylbutanoic acid (as TMS ester). (MS conditions: Ion source 250 °C, ionisation energy 70 eV, mass range 50-550 Daltons).
Complete characterisation of 4-(4'-iso-butylcyclohexyl)butanoic acid by NMR (Figures 2.31 to 2.34) was not possible owing to the complexity of the spectra. This was due to the large number of methylene groups in very similar chemical environments in the two diasteromers of III. Importantly however, it can be seen from the spectra that the aromatic protons and carbons in II were no longer present following hydrogenation to III, whilst the intense doublet (0.84 ppm, integral 6.6H; Figure 2.31) caused by the isopropyl moiety in the iso-butyl substituent remained. An enlarged area of the doublet reveals a smaller doublet slightly up field; this is consistent with the diastereomers observed by GC-MS (Figure 2.24). The multiplet at 2.3 ppm was assigned to the methylenic protons H-10 (integral 2).

The downfield shift relative to the other proton signals is caused by the deshielding effect
of the neighbouring carboxyl moiety. The 17 remaining protons form a complex mix of overlapping multiplets with an integral of 16.1.

Infrared spectroscopy (Figure 2.35) confirmed the loss of aromaticity, while all other functional assignments were the same as those observed for 4-(4'-iso-butylphenyl)butanoic acid (Section 2.4.2).
Figure 2.31. $^1$H NMR spectrum of 4-(4'-iso-butylcyclohexyl)butanoic acid conformers. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform).

Figure 2.32. $^1$H NMR spectra of 4-(4'-iso-butylcyclohexyl)butanoic acid conformers (0.7 and 2.4 ppm). (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform).
Figure 2.33. $^{13}$C NMR spectra of 4-(4'-iso-butylcyclohexyl)butanoic acid conformers. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform).

Figure 2.34. DEPT NMR spectra of 4-(4'-iso-butylcyclohexyl)butanoic acid conformers. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform).
Figure 2.35. Infrared spectrum of 4-(4'-iso-butylecyclohexyl)butanoic acid conformers (KBr discs).

2.4 Synthesis of 4-(4'-butyl-cyclohexyl)butanoic acid

Full experimental details for the synthesis of n-BCHBA can be found in Chapter 5.

2.4.1 Preparation and characterisation of 4-(4'-n-butylphenyl)-4-oxobutanoic acid

4-(4'-n-butylphenyl)-4-oxobutanoic acid (Figure 2.36; IV) was formed in reasonable yield (37%) via a Friedel-Crafts acylation of n-butylbenzene using succinic anhydride, with aluminium chloride as a catalyst, and tetrachloroethane as the solvent. A GC-MS total ion current chromatogram of 4-(4'-n-butylphenyl)-4-oxobutanoic acid as the trimethylsilyl (TMS) ester is represented in Figure 2.37. Integration of the GC peak areas showed the crude keto acid (I) to be >96% pure with no single impurity >1%. Therefore, further purification was deemed unnecessary.

Figure 2.36. Reaction scheme for the synthesis of 4-(4'-n-butylphenyl)-4-oxobutanoic acid (IV).
Confirmation of the structure of IV was provided by mass spectrometry where the molecular ion (M⁺, m/z 306) was observed in the electron impact mass spectrum of the TMS ester (Figure 2.38). The ease of loss of the methyl group of the TMS ester accounts for the relative low intensity of the molecular ion and for the ion at m/z 291 (M⁺ -CH₃), characteristic of TMS esters. The low intensity ion at m/z 263 results from benzylic cleavage of the n-butyl side chain, while the base peak at m/z 161 results from benzylic cleavage to leave a 4-n-butylbenzoyl fragment (Figure 2.38).

![Figure 2.37. Total ion current gas chromatogram analysis of 4-(4'-n-butylphenyl)-4-oxobutanoic acid (as TMS ester). (GC-MS conditions: Column, HP-1MS 30 m x 0.25 mm id x 0.25 μm film; oven programme; 40 – 300 °C at 10 °C min⁻¹, hold 10 min; injector 250 °C).]
Analysis of the keto acid (IV) by $^1$H, $^{13}$C and DEPT NMR spectroscopy (Figures 2.39 to 2.42) led to complete assignment of chemical shifts (Table 2.3). The $^1$H NMR spectrum of 4-(4'-n-butylphenyl)-4-oxobutanoic acid was characterised by a triplet (integral 3H) of resonances at 0.92 ppm assigned to the three protons of the methyl group (H-1). The signal splitting (triplet) was attributed to the effect of two adjacent neighbouring protons (H-2). A multiplet at 1.33 ppm (integral 1.9H) was attributed to H-2, and the multiplicity was expected from the neighbouring methyl protons (H-1) and two adjacent methylenic protons (H-3). The quintet at 1.60 ppm (integral 1.9) was attributed to H-3. The signal splitting was attributed to the two neighbouring methylenic protons (H-2 and H-4) and the downfield shift from H-2 a result of less shielding to the aromatic protons. In turn, the influence of the only two neighbouring protons (H-3) on the splitting of the resonance signal of the methylenic protons (H-4) was consistent with the presence of a triplet (integral 1.8H) at 2.65ppm assigned to H-4. Assignments of H-1, H-2, H-3 and H-4 were further confirmed by the analysis of n-butylbenzene (Appendix 2). This effectively establishes the presence of the n-butyl group substituent (Figure 2.39). The aromatic protons (H-6 and H-7) are
observed as two doublets at 7.26 and 7.88 ppm (integrals of 1.9H and 2.0H, respectively). The two doublets confirm that each aromatic proton has only one neighbouring proton, thus confirming that butanoic keto acid substitution had occurred para to the position of the n-butyl group. The remaining protons are observed as triplets at 2.79 (integral 1.8H) and 3.28 ppm (integral 1.9H) and were assigned to H-11 and H-10, respectively. The triplet at 3.28 ppm was assigned to H-10 as it was considered that the interaction with the neighbouring carbonyl carbon would have a greater downfield effect than that of the interaction of H-11 (2.79 ppm) with the carboxyl group.

The $^{13}$C NMR spectrum of 4-(4'-n-butylphenyl)-4-oxobutanoic acid (Figure 2.41) shows a resonance at 14.0 ppm which remains above the baseline in the DEPT spectrum (Figure 2.42) indicating that it is either a tertiary or a methyl carbon. Thus, the resonance at 14.0 ppm is attributed to the methyl group of the n-butyl moiety (C-1). The resonances represented by peaks below the baseline in the DEPT spectrum (Figure 2.42) are due to secondary methylenic (-CH$_2$-) carbons (C-2, C-3, C-4, C-10 and C-11). C-4 is assigned to the resonance at 35.8 as lack of shielding from the aromatic group causes a downfield shift compared to C-2, C-3, C-10 and C-11. C-2 is assigned to the resonance at 22.38. These assignments are further supported by signals observed in the NMR spectrum of n-butylbenzene (Appendix 2). C-11 is assigned to the resonance at 28.15 ppm while C-10 and C-3 are assigned to those at 33.3 and 33.1 ppm. The carbonyl (C-9) and carboxyl carbons (C-12) show resonances at 197.6 and 178.7 ppm, respectively, and as is characteristic of quaternary carbons, neither are represented by signals on the DEPT spectrum (Williams and Fleming, 1973). The aromatic carbons show resonances between 125 – 150 ppm, of which the resonances at 134.2 and 149.2 ppm are not present on the DEPT spectrum and so are assigned to the substituted quaternary C-5 and C-8. It is likely that the influence of the carbonyl carbon (C-9) has caused the resonance of C-8 to be shifted slightly downfield than that of C-5. The four remaining carbons of the aromatic
ring (C-6 and C-7) are seen as two resonances (128.3 and 128.8 ppm), which provides further evidence that the compound is para disubstituted. The slight downfield shift of C-7 compared to that of C-6 is due to the interaction between C-7 and the carbonyl carbon C9.
Figure 2.39. $^1$H NMR spectrum of 4-(4'-n-butylphenyl)-4-oxobutanoic acid. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform. Table 2.3 contains details of chemical shifts and integrals).

Figure 2.40. $^1$H NMR spectrum of 4-(4'-n-butylphenyl)-4-oxobutanoic acid, expanded region between 0.5 and 3.5 ppm. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform. Table 2.3 for chemical shifts and integrals).
Figure 2.41. $^{13}$C NMR spectrum of 4-(4'-n-butylyphenyl)-4-oxobutanoic acid. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform. Table 2.3 contains details of chemical shifts and integrals).

Figure 2.42. $^{13}$C DEPT NMR spectra of 4-(4'-n-butylyphenyl)-4-oxobutanoic acid. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform. Table 2.3 contains details of chemical shifts and integrals).
Table 2.3. Assignment for $^1$H and $^{13}$C resonances in NMR spectra of 4-(4'-n-butylphenyl)-4-oxobutanoic acid.

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$^1$ Assignments for C-3 and C-10 may be interchanged.

Infrared spectroscopic examination of 4-(4'-n-butylphenyl)-4-oxobutanoic acid (Figure 2.43) showed a characteristic broad absorption band at 3434 cm$^{-1}$ (stretching) due to the hydrogen bonded carboxylic acid –OH group. Two peaks at 1697 cm$^{-1}$ and 1678 cm$^{-1}$ are assigned to the carboxyl and carbonyl stretching respectively. Strong bands at 2956 and 2862 cm$^{-1}$ were assigned to methylenic and methyl C-H bond stretching. The sharp bands at 1605 and 1569 cm$^{-1}$ were assigned to aromatic ring vibrations, whilst the sharp band at 828 cm$^{-1}$ is characteristic of para-disubstitution. Other absorptions observed in the fingerprint region were likely to be due to aliphatic C-H vibrations (~1350 – 1500 cm$^{-1}$) and the C-O stretch of the carboxylic acid (~1200 – 1300 cm$^{-1}$).
2.4.2 Preparation and characterisation of 4-(4'-n-butylylphenyl)butanoic acid

4-(4'-n-butylylphenyl)-4-oxobutanoic acid (IV) was converted into 4-(4'-n-butylylphenyl)butanoic acid (V) via a Huang-Minlon modification of a Wolff-Kischner reduction (Huang-Minlon, 1946) using hydrazine hydrate with potassium hydroxide and ethylene glycol as solvent (Figure 2.44). Acid (V) was obtained in excellent yield (84%) and purity (>96%) as shown by GC-MS analysis of the TMS ester (Figure 2.45).

Figure 2.44. Reaction scheme for the synthesis of 4-(4'-n-butylylphenyl)butanoic acid (V).

The mass spectrum (Figure 2.46) contained a molecular ion at \( m/z \) 292, and an ion at \( m/z \) 277, which corresponds to \( M^+ - 15 \) (i.e. the loss of a methyl group). The ion at \( m/z \) 160 was the result of \( \gamma \)-H rearrangement known as the McLafferty rearrangement which occurs in compounds containing an unsaturated functionality, the carbonyl group in this case, along
with a \( y\)-H (McLafferty and Tureček, 1993). Cleavage at the esteric carbon results in an ion at \( m/z \) 117 (\(^{1}C\)OOTTMS), characteristic of TMS esters.

Figure 2.45. Total ion current gas chromatogram of 4-\( (4'\)-n-butylphenyl)butanoic acid (as TMS ester). (GC-MS conditions: Column, HP-1MS 30 m x 0.25 mm id x 0.25 \( µ \)m film; oven programme: 40 – 300 °C at 10 °C min\(^{-1}\), hold 10 min; injector 250 °C).

Figure 2.46. Mass spectrum of 4-\( (4'\)-n-butylphenyl)butanoic acid (as TMS ester). (MS conditions: Ion source 250 °C, ionisation energy 70 eV, mass range 50 -550 Daltons).
Analysis of II by $^1$H, $^{13}$C and DEPT NMR spectroscopy (Figures 2.47 to 2.51) led to complete assignment of proton and carbon chemical shifts (Table 2.4). Reduction of the keto group had very little effect on the protons in the $n$-butyl side chain, thus these resonances are assigned as described in the previous section (H-1 0.92 ppm, H-2 1.36 ppm, H-3 1.58 ppm and H-4 2.63 ppm; section 2.5.1). The proton spectrum also shows that the aromatic protons are now magnetically equivalent, thus observed as a singlet (7.09 ppm; integral 3.9H). A triplet at 2.57 ppm (integral 2H) was assigned to the methylenic protons at H-9 which result from the reduction of the carbonyl group. As expected, the chemical shift was very similar to that of H-4 because both protons are adjacent to the aromatic ring. The multiplet at 1.95 ppm (integral 1.9H) was attributed to H-10, and the multiplicity was as expected from the presence of four adjacent methylenic protons (H-9 and H-11). The remaining triplet at 2.37 ppm (integral 1.8H) was attributed to H-11. The notable differences in the $^{13}$C and DEPT spectra (Figure 2.47 to 2.52) to those of 4-(4'-$n$-butylphenyl)-4-oxobutanoic acid (Figure 2.42) are the loss of the carbonyl resonance at 197.6 ppm and the addition of a methylenic resonance at 34.5 ppm (C-9).
Figure 2.47. $^1$H NMR spectrum of 4-(4'-$n$-butylphenyl)butanoic acid. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform. Table 2.4 contains details of chemical shifts and integral).

Figure 2.48. $^1$H NMR spectrum of 4-(4'-$n$-butylphenyl)butanoic acid, expanded region between 0.5 and 3.0 ppm. (Joel EX270MHz HR FT-NMR. Samples dissolved in deuterated chloroform. Table 2.4 contains chemical shifts and integrals).
Figure 2.49. $^{13}$C NMR spectrum of 4-(4'-n-butylphenyl)butanoic acid. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform. Table 2.4 contains chemical shifts).

Figure 2.50. DEPT NMR spectrum of 4-(4'-n-butylphenyl)butanoic acid. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform, Table 2.4 contains chemical shifts).
Figure 2.51. DEPT NMR spectrum of 4-(4'-n-butyphenyl)butanoic acid, expanded region between 10 and 40 ppm. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform, Table 2.4 contains chemical shifts).

Table 2.4. Assignment of $^1$H and $^{13}$C resonances in NMR spectra of 4-(4'-n-butyphenyl)butanoic acid.

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<th>J (Hz)</th>
<th>$^{13}$C chemical shift (ppm)</th>
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</table>

$^1$ Protons for 6 and 7 are in identical environments.
$^2$ Assignments of C5 and C8 may be interchanged.
$^3$ Assignments of C6 and C7 may be interchanged.
$^4$ H-4 and H-9 have been integrated together as they are not resolved.
The infrared spectrum of 4-(4'-n-butylphenyl)butanoic acid (Figure 2.52) was very similar to that described for 4-(4'-n-butylphenyl)-4-oxobutanoic acid (Section 2.5.1) with the notable difference that the absorbance due to the C=O in the keto group was now absent in the spectrum of V.

![Infrared spectrum of 4-(4'-n-butylphenyl)butanoic acid (KBr disc).](image)

**Figure 2.52.** Infrared spectrum of 4-(4'-n-butylphenyl)butanoic acid (KBr disc).

### 2.4.3 Preparation and characterisation of 4-(4'-n-butylecyclohexyl)butanoic acid

4-(4'-n-butylphenyl)butanoic acid (V) was converted into 4-(4'-n-butylecyclohexyl)butanoic acid (Figure 2.53; VI) via a hydrogenation reaction adapted from Dauben and Hoerger (1951), using platinum oxide as the catalyst and acetic acid as the solvent with a steady stream of hydrogen under pressure (ca. 8 psi; ca. 6 hours). 4-(4'-n-butylecyclohexyl)butanoic acid was obtained in quantitative amounts (ca. 200 mg) and high purity (>96 %) as two isomers shown by GC-MS analysis of the TMS ester (Figure 2.54).

![Reaction scheme for hydrogenation of 4-(4'-n-butylphenyl)butanoic acid (V) to the surrogate naphthenic acid 4-(4'-n-butylecyclohexyl)butanoic acid (n-BCHBA; VI).](image)

**Figure 2.53.** Reaction scheme for hydrogenation of 4-(4'-n-butylphenyl)butanoic acid (V) to the surrogate naphthenic acid 4-(4'-n-butylecyclohexyl)butanoic acid (n-BCHBA; VI).
Mass spectrometry, in conjunction with NMR and IR spectroscopy, confirmed the structure of 4-(4'-n-butylcyclohexyl)butanoic acid. The mass spectra (Figure 2.55) of both isomers were identical, each exhibiting a molecular ion of \( m/z \) 298 (as TMS esters) as expected. Both the loss of a methyl \( (m/z \) 283; \( M^+ - 15 \)) and a base peak ion of \( m/z \) 117 are characteristic of TMS esters of carboxylic acids. The ions observed at \( m/z \) 255 \( (M^+ - 43) \) and \( m/z \) 239 \( (M^+ - 59) \) are characteristic of rearrangements for butanoic TMS ester substituted cyclohexyl moieties and are discussed fully for iso-BCHBA in section 4.4.3.

**Figure 2.54.** Total ion current gas chromatogram of 4-(4'-n-butylcyclohexyl)butanoic acid conformers (as TMS ester). Inset, expanded region of chromatogram 16 - 17.2 min. (GC-MS conditions: Column, HP-1MS 30 m x 0.25 mm id x 0.25 μm film; oven programme; 40 – 300 °C at 10 °C min\(^{-1}\), hold 10 min; injector 250 °C, ion source 280 °C, 70 eV).
Figure 2.55. Mass spectrum of 4-(4'-n-butylcyclohexyl)butanoic acid (as TMS ester). (MS conditions: Ion source 250 °C, ionisation energy 70 eV, mass range 50 -550 Daltons).

Complete characterisation of 4-(4'-n-butylcyclohexyl)butanoic acid by NMR spectroscopy (Figure 2.56 to 2.69) was not possible due to the complexity of the spectra. This was due to the large number of methylene groups in very similar chemical environments in the two diastereomers of VI. However, importantly, it can be seen from the spectra that the aromatic protons and carbons in V (Figure 2.47) were no longer present following hydrogenation to VI. The triplet at 0.8 ppm (Figure 2.56; integral 4.2H) has been assigned to the methyl protons H-1. An enlarged area of the triplet reveals another triplet slightly up field; this is consistent with the diastereomers observed by GC-MS (Figure 2.54). It is likely that the observed integral of 4.2 is above the expected 3 because of integration errors associated with lack of baseline separation between the multiplets. The multiplet at 2.33 ppm has been assigned to the methylenic protons H-11 (integral 2H). The downfield shift relative to the other proton signals is caused by the deshielding effect of the neighbouring carboxyl moiety. The 20 remaining protons form a complex mix of overlapping multiplets with an integral of 20H.
Infrared spectroscopy (Figure 2.60) confirmed the loss of aromaticity, while all other functional assignments were the same as those observed for 4-(4'-n-butylphenyl)butanoic acid.
Figure 2.56. $^1$H NMR spectrum of 4-(4′-n-butylocyclohexyl)butanoic acid. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform).

Figure 2.57. $^1$H NMR spectra of 4-(4′-n-butylocyclohexyl)butanoic (0.7 and 2.4 ppm). (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform).
**Figure 2.58.** $^{13}$C NMR spectra of 4-(4′-n-butylocyclohexyl)butanoic acid. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform).

**Figure 2.59.** DEPT NMR spectra of 4-(4′-n-butylocyclohexyl)butanoic acid. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform).
2.5 Synthesis of 4-(4'-sec-butyl-cyclohexyl)butanoic acid

Full experimental details for the synthesis of sec-BCHBA can be found in Chapter 5.

2.5.1 Preparation and characterisation of 4-(4'-sec-butylphenyl)-4-oxobutanoic acid

4-(4'-sec-butylphenyl)-4-oxobutanoic acid (Figure 2.61; I) was synthesised in reasonable yield (39%) via a Friedel-Crafts acylation of sec-butylbenzene using succinic anhydride, with aluminium chloride as a catalyst, and tetrachloroethane as the solvent.

![Figure 2.61. Reaction scheme for the synthesis of 4-(4'-sec-butylphenyl)-4-oxobutanoic acid (VII).](image)

The GC-MS total ion current chromatogram of 4-(4'-sec-butylphenyl)-4-oxobutanoic acid as the trimethylsilyl (TMS) ester is presented in Figure 2.62. Integration of the GC peak areas shows the crude keto acid (VII) to be of reasonable purity >94%. Confirmation of the
structure of VII was provided by mass spectrometry where the molecular ion \((M^+, \text{ } m/z \text{ } 306)\) was observed in the electron impact mass spectrum of the TMS ester (Figure 2.63). The ease of loss of the methyl group of the TMS ester accounts for the relative low intensity of the molecular ion and for the ion at \(m/z \text{ } 291\) \((M^+ - \text{CH}_3)\), characteristic of TMS esters, while the base peak at \(m/z \text{ } 161\) results from benzylic cleavage to leave a 4-sec-butylbenzoyl fragment (Figure 2.63).

![Figure 2.62. Total ion current gas chromatogram of 4-(4'-sec-butylphenyl)-4-oxobutanoic acid (as TMS ester). (GC-MS conditions: Column, HP-1MS 30 m x 0.25 mm id x 0.25 μm film; oven programme; 40 - 300 °C at 10 °C min\(^{-1}\), hold 10 min; injector 250 °C).](image)
Analysis of the keto acid (VII) by $^1$H, $^{13}$C and DEPT NMR spectroscopy (Figures 2.64 to 2.67) led to complete assignment of chemical shifts (Table 2.5). The $^1$H NMR spectrum of 4-(4'-sec-butylphenyl)-4-oxobutanoic acid was characterised by a triplet (integral 3H) of resonances at 0.79 ppm assigned to the three protons of the methyl group (H-1). The signal splitting (triplet) was attributed to the effect of two adjacent neighbouring protons (H-2). The doublet at 1.20 ppm (integral 3H) was assigned to H-4, the signal splitting attributed to one neighbouring proton (H-3). The quintet at 1.58 ppm (integral 2H) was attributed to H-2, and the multiplicity was expected from the neighbouring methyl protons (H-1) and the adjacent single proton (H-3). The overlapping multiplet at 2.61 ppm with the triplet at 2.70 ppm has been assigned to H-3 while the triplet is assigned to H-11. The integral for the multiplet and triplet was 3, as expected. The triplet at 3.24 ppm has been assigned to H-9 (integral 1.8) with the signal splitting caused by the adjacent methylenic protons (H-11) and the relative downfield shift caused by the deshielding to the adjacent carbonyl group. Analysis of sec-butylbenzene (data not shown) gave further confirmation of H-1, H-2, H-3 and H-4 assignments. This effectively establishes the presence of the sec-butyl group.
substituent (Figure 2.64). The aromatic protons (H-6 and H-7) were observed as two doublets at 7.20 and 7.85 ppm, respectively (integrals of 2.0H and 2.1H, respectively). The two doublets confirm that each aromatic proton has only one neighbouring proton, thus confirming that butanoic keto acid substitution had occurred para to the position of the sec-butyl group.

The $^{13}$C NMR spectrum of 4-(4'-sec-butylphenyl)-4-oxobutanoic acid (Figure 2.66) shows a relatively intense resonance at 12.2 ppm which remains above the baseline in the DEPT spectrum (Figure 2.67) indicating that it is due to either a tertiary or a methyl carbon. Thus, the resonance at 14.0 ppm is attributed to the methyl group C-1 of the sec-butyl moiety as it has the most shielding from the aromatic protons causing the greatest upfield shift. The resonances at 21.6 and 41.9 ppm also remain above the baseline in the DEPT spectrum and have been attributed to C-4 and C-3, respectively. The resonances represented by peaks below the baseline between 25-40 ppm in the DEPT spectrum (Figure 2.67) are due to secondary methylenic carbons (C-2, C-10 and C-1). Analysis of sec-butylbenzene (data not shown) confirmed C-2 assignment to the resonance at 31.0, whilst C-10 and C-11 were assigned to the resonances at 33.6 and 28.7 ppm, respectively. C-10 was assigned to 33.6 ppm as the adjacent carbonyl group causes a greater downfield shift than the adjacent carboxyl group to C-11. The carbonyl (C-9) and carboxyl (C-12) carbons resonances were at 197.6 and 178.7 ppm, respectively, and are characteristic of quaternary carbons. Neither were represented by signals on the DEPT spectrum (Williams and Fleming, 1973). The aromatic carbon resonances were observed between 125 – 150 ppm, of which the resonances at 134.5 and 153.8 ppm were not present on the DEPT spectrum and so were assigned to the substituted quaternary C-5 and C-8 carbons, respectively. It is likely that the influence of the carbonyl carbon (C-9) caused the resonance of C-8 to be shifted slightly downfield to that of C-5. The four remaining carbons of the aromatic ring were seen as two resonances (128.4 and 127.4 ppm), which provides further evidence that the
compound is para disubstituted. The proximity of the carbonyl carbon to C7 (128.4 ppm) caused a slight downfield shift relative to C6 (127.4 ppm).

Figure 2.64. $^1$H NMR spectrum of 4-(4'-sec-butylphenyl)-4-oxobutanoic acid. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform). Table 2.5 contains details of chemical shifts and integrals.

Figure 2.65. $^1$H NMR spectrum of 4-(4'-sec-butylphenyl)-4-oxobutanoic acid, expanded region between 0.5 and 3.5 ppm. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform). Table 2.5 contains details of chemical shifts and integrals.
Figure 2.66. $^{13}$C NMR spectrum of 4-(4'-sec-butylphenyl)-4-oxobutanoic acid. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform). Table 2.5 contains details of chemical shifts and integrals.

Figure 2.67. $^{13}$C DEPT NMR spectra of 4-(4'-sec-butylphenyl)-4-oxobutanoic acid. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform). Table 2.5 contains details of chemical shifts and integrals.
Table 2.5. Assignment for $^1$H and $^{13}$C resonances in NMR spectra of 4-(4'-sec-butyphenyl)-4-oxobutanoic acid.

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$^1$H-3 and H-11 have been integrated together as they are not resolved.

Infrared spectroscopic examination of 4-(4'-sec-butyphenyl)-4-oxobutanoic acid (Figure 2.68) showed a characteristic broad absorption band at 3452 cm$^{-1}$ due to the hydrogen bonded carboxylic acid $-$OH group. A band resulting from two peaks at 1716 cm$^{-1}$ and 1685 cm$^{-1}$ are assigned to the carboxyl and carbonyl stretching respectively. Strong bands at 2964 and 2923 cm$^{-1}$ were assigned to methylenic and methyl C-H bond stretching. The sharp bands at 1606 and 1567 cm$^{-1}$ were assigned to aromatic ring vibrations, whilst the sharp band at 825 cm$^{-1}$ is characteristic of $para$-disubstitution. Other absorptions observed in the fingerprint region were likely due to aliphatic C-H bending ($\sim 1350 - 1500$ cm$^{-1}$) and the C-O stretch of the carboxylic acid ($\sim 1200 - 1300$ cm$^{-1}$).
2.5.2 Preparation and characterisation of 4-(4'-sec-butylphenyl)butanoic acid

4-(4'-sec-butylphenyl)-4-oxobutanoic acid (VII) was converted into 4-(4'-sec-butylphenyl)butanoic acid (VIII) via a Huang-Minlon modification of a Wolff-Kischner reduction (Huang-Minlon, 1946) using hydrazine hydrate with potassium hydroxide and ethylene glycol as solvent (Figure 2.69). Acid (VIII) was obtained in excellent yield (97%) and reasonable purity (>93%) as shown by GC-MS analysis of the TMS ester (Figure 2.70), with the major impurity being residual ethylene glycol.

![Infrared spectrum of 4-(4'-sec-butylphenyl)-4-oxobutanoic acid (KBr disc).](image)

Figure 2.68. Infrared spectrum of 4-(4'-sec-butylphenyl)-4-oxobutanoic acid (KBr disc).

The mass spectrum (Figure 2.71) contained a molecular ion at $m/z$ 292, and an ion at $m/z$ 277, which corresponds to $M^+ - 15$ (i.e. loss of a methyl group). The ion at $m/z$ 160 was the
result of γ-H rearrangement known as the McLafferty rearrangement which occurs in compounds containing an unsaturated functionality, the carbonyl group in this case, along with a γ-H (McLafferty and Tureček, 1993). Cleavage at the esteric carbon results in an ion at m/z 117 (COOTMS) which is characteristic of TMS esters. The ion at m/z 263 results from benzylic cleavage at the branch point of the sec-butyl side chain.

Figure 2. Total ion current gas chromatogram of 4-(4′-sec-butylphenyl)butanoic acid (as TMS ester). (GC-MS conditions: Column, HP-1MS 30 m x 0.25 mm id x 0.25 μm film; oven programme; 40 – 300 °C at 10 °C min⁻¹, hold 10 min; injector 250 °C).
Figure 2.71. Mass spectrum of 4-(4'-sec-butylphenyl)butanoic acid (as TMS ester). (MS conditions: ion source 250 °C, ionisation energy 70 eV, mass range 50 - 550 Daltons).

Analysis of II by $^1$H, $^{13}$C and DEPT NMR spectroscopy (Figures 2.72 to 2.76) led to complete assignment of proton and carbon chemical shifts (Table 2.6). Reduction of the keto group had little effect on the protons in the sec-buty1 side chain, thus the resonances were assigned as described in the previous section (H-1 0.81 ppm, H-2 1.57 ppm, H-3 2.57 ppm and H-4 1.22 ppm; section 2.6.1). The proton spectrum also showed that the aromatic protons (H-6 and H-7) were now magnetically equivalent and now observed as a singlet (7.09 ppm; integral 4.0H). A triplet at 2.63 ppm was assigned the methylenic protons at H-9 which result from the reduction of the carbonyl group. As expected, the chemical shift was very similar to that of H-3 because both protons are adjacent to the aromatic ring, as a consequence both signals were integrated together (integral 2.8H). The multiplet at 1.95 ppm (integral 1.8H) was attributed to H-10, and the multiplicity was as expected from the presence of four adjacent methylenic protons (H-9 and H-11). The remaining triplet at 2.37 ppm (integral 1.8H) was attributed to H-11. The notable differences in the $^{13}$C and DEPT
spectra to those of 4-(4'-sec-butylphenyl)-4-oxobutanoic acid were the loss of the carbonyl resonance at 198.2 ppm and the addition of a methylenic resonance at 34.7 ppm (C-9).

Two small multiplets were present in the in the $^1$H spectrum at 3.77 and 3.62 ppm (integral 0.7) which were due to contamination. These contaminants were also observed in the $^{13}$C spectrum at 72.4 and 61.8 ppm. Their presence below the baseline in the DEPT spectrum indicates that they contained methylenic carbons and are thought to arise from residual ethylene glycol.
Figure 2.72. $^1$H NMR spectrum of 4-(4'-sec-butylphenyl)butanoic acid. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform. Table 2.6 contains details of chemical shifts and integral).

Figure 2.73. $^1$H NMR spectrum of 4-(4'-sec-butylphenyl)butanoic acid, expanded region between 0.5 and 3.0 ppm. (Joel EX270MHz HR FT-NMR. Samples dissolved in deuterated chloroform. Table 2.6 contains chemical shifts and integrals).
Figure 2.74. $^{13}$C NMR spectrum of 4-(4'-sec-butylphenyl)butanoic acid. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform. Table 2.6 contains chemical shifts).

Figure 2.75. DEPT NMR spectrum of 4-(4'-sec-butylphenyl)butanoic acid. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform. Table 2.6 contains chemical shifts).
Figure 2.76. DEPT NMR spectrum of 4-(4'-sec-butylphenyl)butanoic acid, expanded region between 10 and 45 ppm. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform. Table 2.6 contains chemical shifts).

Table 2.6. Assignment of $^1$H and $^{13}$C resonances in NMR spectra of 4-(4'-sec-butylphenyl)butanoic acid.

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<th>$^{13}$C chemical shift (ppm)</th>
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$^1$ Protons for 6 and 7 are magnetically equivalent.
$^2$ Assignments of C5 and C8 may be interchanged.
$^3$ Assignments of C6 and C7 may be interchanged.
$^4$ H-3 and H-9 have been integrated together as they are not resolved.
The infrared spectrum (Figure 2.77) was very similar to that described for 4-(4'-sec-butylphenyl)-4-oxobutanoic acid in the previous section with the notable difference that the absorbance due to the C=O in the keto group (1685 cm⁻¹) was now absent in the spectrum of VIII.

![Infrared spectrum of 4-(4'-sec-butylphenyl)butanoic acid (KBr discs).](image)

**Figure 2.77.** Infrared spectrum of 4-(4'-sec-butylphenyl)butanoic acid (KBr discs).

### 2.5.3 Preparation and characterisation of 4-(4'-sec-butylcyclohexyl)butanoic acid

4-(4'-sec-butylphenyl)butanoic acid (VIII) was converted into 4-(4'-sec-butylcyclohexyl)butanoic acid (Figure 2.78; IX) via a hydrogenation reaction adapted from Dauben and Hoerger (1951), using platinum oxide as the catalyst and acetic acid as the solvent with a steady stream of hydrogen under pressure (ca. 8 psi; ca. 6 hours). 4-(4'-sec-butylcyclohexyl)butanoic acid was obtained in quantitative amounts (ca. 200 mg) and high purity (>94%) as two isomers shown by GC-MS analysis of the TMS ester (Figure 2.79).
Mass spectrometry, in conjunction with NMR and IR spectroscopy, confirmed the structure of 4-(4'-sec-butylecyclohexyl)butanoic acid. The mass spectra (Figure 2.80) of both isomers were identical, each exhibiting a molecular ion of $m/z$ 298 (as TMS ester) as expected. Methyl loss ($m/z$ 283; $M^+ - 15$) and a base peak ion of $m/z$ 117 are characteristic of mass spectra of TMS esters of carboxylic acids. The ions observed at $m/z$ 255 ($M^+ - 43$) and $m/z$ 239 ($M^+ - 59$) are characteristic of rearrangements for butanoic TMS ester substituted cyclohexyl moieties and are discussed more fully for iso-BCHBA in section 2.4.3.

Figure 2.79. Total ion current gas chromatogram of 4-(4'-sec-butylecyclohexyl)butanoic acid isomers (as TMS ester). Inset, expanded region of chromatogram 15.8 - 17.0 min. (GC-MS conditions: Column, HP-1MS 30 m x 0.25 mm id x 0.25 µm film; oven programme; 40 - 300 °C at 10 °C min$^{-1}$, hold 10 min; injector 250 °C, ion source 280 °C, 70 eV).
Complete characterisation of 4-(4'-sec-butylcyclohexyl)butanoic acid by NMR spectroscopy (Figures 2.81 to 2.84) was not possible due to the complexity of the spectra. This was due to the large number of methylene groups in very similar chemical environments in the two diasteromers of III. Importantly however, it can be seen from the spectra that the aromatic protons and carbons in II were no longer present following hydrogenation to III. The proton multiplet at 0.82 ppm (Figure 2.81; integral 6.7H) has been assigned to the methyl protons H-1 and H-4. It is likely that the observed integral of 6.7 is above the expected 6 protons because of integration errors associated with incomplete baseline separation between the multiplets. The multiplet at 2.33 ppm has been assigned to the methylenic protons H-11 (integral 2H). The downfield shift relative to the other proton signals is caused by the deshielding effect of the neighbouring carboxyl moiety. The 17 remaining protons form a complex mix of overlapping multiplets with an integral of 17.1H.
Infrared spectroscopy (Figure 2.85) confirmed the loss of aromaticity (absence of absorbances at 3010 and 1512 cm$^{-1}$), while all other functional assignments were the same as those observed for 4-(4'-sec-butylphenyl)butanoic acid.
Figure 2.81. $^1$H NMR spectrum of 4-(4'-sec-butylcyclohexyl)butanoic acid. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform).

Figure 2.82. $^1$H NMR spectrum of 4-(4'-sec-butylcyclohexyl)butanoic acid focused (0.5 and 2.5 ppm). (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform).
Figure 2.83. $^{13}$C NMR spectrum of 4-(4'-sec-butylcyclohexyl)butanoic acid. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform).

Figure 2.84. DEPT NMR spectrum of 4-(4'-sec-butylcyclohexyl)butanoic acid. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform).
2.6 Synthesis of 4-(4'-tert-butyl-cyclohexyl)butanoic acid

Full experimental details for the synthesis of tert-BCHBA can be found in Chapter 5.

2.6.1 Preparation and characterisation of 4-(4'-tert-butylphenyl)-4-oxobutanoic acid

4-(4'-tert-butylphenyl)-4-oxobutanoic acid (Figure 2.86; X) was formed in excellent yield (75 %) via a Friedel-Crafts acylation of tert-butylbenzene using succinic anhydride, with aluminium chloride as a catalyst, and tetrachloroethane as the solvent. Figure 2.87 shows a GC-MS total ion current chromatogram of 4-(4'-tert-butylphenyl)-4-oxobutanoic acid as the trimethylsilyl (TMS) ester. Integration of the GC peak areas showed the crude keto acid (I) to be >95% pure. No further purification was deemed necessary.

![Figure 2.86. Reaction scheme for the synthesis of 4-(4'-tert-butylphenyl)-4-oxobutanoic acid (I).](image-url)
Confirmation of the structure of X was provided by mass spectrometry where the molecular ion \((M^+, m/z 306)\) was observed in the electron impact mass spectrum of the TMS ester (Figure 2.87). The ease of loss of the methyl group of the TMS ester accounts for the relatively low intensity of the molecular ion and for the ion at \(m/z 291 (M^- -\text{CH}_3)\), characteristic of TMS esters. The base peak at \(m/z 161\) results from benzylic cleavage to leave a 4-tert-butylbenzoyl fragment (Figure 2.98).

**Figure 2.87.** Total ion current gas chromatogram analysis of 4-(4'-tert-butylphenyl)-4-oxobutanoic acid (as TMS ester). (GC-MS conditions: Column, HP-1MS 30 m x 0.25 mm id x 0.25 μm film; oven programme; 40 – 300 °C at 10 °C min\(^{-1}\), hold 10 min; injector 250 °C).
Figure 2.88. Mass spectrum of 4-(4'-tert-butylphenyl)-4-oxobutanoic acid (as TMS ester). (MS conditions: ion source 250 °C, ionisation energy 70 eV, mass range 50 -550 Daltons).

Analysis of the keto acid (X) by $^1$H, $^{13}$C and DEPT NMR spectroscopy (Figures 2.90 to 2.93) led to complete assignment of chemical shifts (Table 2.7). The $^1$H NMR spectrum of 4-(4'-tert-butylphenyl)-4-oxobutanoic acid was characterised by an intense single resonance (singlet; integral 9.1H) 1.33 ppm assigned to the nine protons of the three magnetically equivalent methyl groups (H-1). The intense singlet was expected as the three methyl groups are attached to a quaternary carbon. The singlet effectively establishes the presence of the tert-butyl moiety. The aromatic protons (H-4 and H-5) are observed as two doublets at 7.47 and 7.91 ppm, respectively (integrals of 1.9H and 2.0H, respectively). The two doublets confirm that each aromatic proton has only one neighbouring proton, thus confirming that butanoic keto acid substitution had occurred para to the position of the -tert-butyl group. The remaining protons observed as triplets at 2.79 (integral 2.0H) and 3.29 ppm (integral 2.0H) and were assigned to H-9 and H-8, respectively. The triplet at 3.29 ppm was assigned to H-8 as it was considered that the interaction with the neighbouring carbonyl carbon would have a greater downfield effect than that of the interaction of the carboxyl group with the neighbouring proton H-9.
The $^{13}$C NMR spectrum of 4-(4′-tert-butylphenyl)-4-oxobutanoic acid (Figure 2.91) shows an intense resonance at 31.2 ppm which remains above the baseline in the DEPT spectrum (Figure 2.92) indicating that it is due either to a tertiary or methyl carbon. Thus, the resonance at 31.2 ppm is attributed to the three magnetically equivalent methyl groups of the tert-butyl moiety (C-1). The resonances represented by peaks below the baseline in the DEPT spectrum are due to secondary methylenic (-CH$_2$-) carbons (C-8 and C-9). C-9 is assigned to the resonance at 28.2 ppm whilst C-8 is assigned to the resonance at 33.1 ppm. Resonances that are present in the $^{13}$C spectrum but not on the DEPT spectrum are quaternary carbons; thus the resonance at 35.2 on the $^{13}$C spectrum has been assigned to C-2. The carbonyl (C-7) and carboxyl carbons (C-10) were assigned to resonances at 197.6 and 178.8 ppm respectively, and as is characteristic of quaternary carbons, neither were represented by signals on the DEPT spectrum (Williams and Fleming, 1973). The aromatic carbons show resonances between 125 – 150 ppm, of which the resonances at 133.9 and 157.8 ppm are not present on the DEPT spectrum and so are due to the substituted quaternary carbons at C-3 and C-6. It is likely that the influence of the carbonyl carbon (C-7) has caused the resonance of C-6 to be slightly downfield relative to that of C-3. The four remaining carbons of the aromatic ring are seen as two resonances (128.1 and 125.7 ppm), which provides further evidence that the compound is para disubstituted. The proximity of the carbonyl carbon to C5 (128.1) caused a slight downfield shift relative to C4 (125.7).
Figure 2.89. $^1$H NMR spectrum of 4-(4'-tert-butylphenyl)-4-oxobutanoic acid. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform. Table 2.7 contains details of chemical shifts and integrals).

Figure 2.90. $^1$H NMR spectrum of 4-(4'-tert-butylphenyl)-4-oxobutanoic acid, expanded region between 1.0 and 3.5 ppm. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform. Table 2.7 for chemical shifts and integrals).
Figure 2.91. $^{13}$C NMR spectrum of 4-(4'-tert-butylphenyl)-4-oxobutanoic acid. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform. Table 2.7 contains details of chemical shifts and integrals).

Figure 2.92. $^{13}$C DEPT NMR spectra of 4-(4'-tert-butylphenyl)-4-oxobutanoic acid. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform. Table 2.7 contains details of chemical shifts and integrals).
Table 2.7. Assignment for $^1$H and $^{13}$C resonances in NMR spectra of 4-(4'-tert-butylphenyl)-4-oxobutanoic acid.

<table>
<thead>
<tr>
<th>Carbon number</th>
<th>$^1$H chemical shift (Integral)</th>
<th>J (Hz)</th>
<th>$^{13}$C chemical shift (ppm)</th>
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<td></td>
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<td></td>
<td>CH$_3$</td>
</tr>
<tr>
<td>1</td>
<td>1.33 (9.1)</td>
<td></td>
<td>31.2</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>7.47 (1.9)</td>
<td>10.8</td>
<td>125.7</td>
</tr>
<tr>
<td>5</td>
<td>7.92 (2.0)</td>
<td>8.1</td>
<td>128.1</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>3.29 (2.0)</td>
<td>8.1</td>
<td>33.1</td>
</tr>
<tr>
<td>9</td>
<td>2.79 (2.0)</td>
<td>8.1</td>
<td>28.1</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Infrared spectroscopic examination of 4-(4'-tert-butylphenyl)-4-oxobutanoic acid (Figure 2.93) showed a characteristic broad absorption band at 3436 cm$^{-1}$ due to the hydrogen bonded carboxylic acid $\text{-OH}$ group. A band resulting from two peaks at 1708 cm$^{-1}$ and 1686 cm$^{-1}$ are assigned to the carboxyl and carbonyl stretching respectively. Strong bands at 2963 and 2867 cm$^{-1}$ were assigned to methylenic and methyl C-H bond stretching. The sharp bands at 1605 and 1567 cm$^{-1}$ were assigned to aromatic ring vibrations, whilst the sharp band at 825 cm$^{-1}$ is characteristic of para-disubstitution. Other absorptions observed in the fingerprint region were likely to be due to aliphatic C-H bending ($\sim$1350 – 1500 cm$^{-1}$) and the C-O stretch of the carboxylic acid ($\sim$1200 – 1300 cm$^{-1}$).
2.6.2 Preparation and characterisation of 4-(4'-tert-butylphenyl)butanoic acid

4-(4'-tert-butylphenyl)-4-oxobutanoic acid (X) was converted into 4-(4'-tert-butylphenyl)butanoic acid (XI) via a Huang-Minlon modification of a Wolff-Kischner reduction (Huang-Minlon, 1946) using hydrazine hydrate with potassium hydroxide and ethylene glycol as solvent (Figure 2.94). Acid (XI) was obtained in excellent yield (>98%) and reasonable purity (>92%) as shown by GC-MS analysis of the TMS ester (Figure 2.95).

The mass spectrum (Figure 2.96) contained a molecular ion at m/z 292, and an ion at m/z 277, which corresponds to M\(^+\) - 15 (i.e. the loss of a methyl group). The ion at m/z 160 was
the result of \( \gamma \)-H rearrangement known as the McLafferty rearrangement which occurs in compounds containing an unsaturated functionality, the carbonyl group in this case, along with a \( \gamma \)-H (McLafferty and Tureček, 1993). Cleavage at the esteric carbon results in an ion at \( m/z \ 117 \) (\(^1{}^{1}COOTMS\)) which is characteristic of TMS esters.

Figure 2.95. Total ion current gas chromatogram of 4-(4’-tert-butylphenyl)butanoic acid (as TMS ester). (GC-MS conditions: Column, HP-1MS 30 m x 0.25 mm id x 0.25 \( \mu \)m film; oven programme; 40 – 300 °C at 10 °C min\(^{-1}\), hold 10 min; injector 250 °C).
Figure 2.96. Mass spectrum of 4-(4′-tert-butylphenyl)butanoic acid (as TMS ester). (MS conditions: Ion source 250 °C, ionisation energy 70 eV, mass range 50-550 Daltons).

Analysis of XI by $^1$H, $^{13}$C and DEPT NMR spectroscopy (Figures 2.98 to 2.101) led to complete assignment of proton and carbon chemical shifts (Table 2.8). Reduction of the keto group had very little effect on the protons in the tert-butyl side chain, thus these resonances were assigned as described in the previous section (H-1 1.29; Section 2.7.1).

The triplet at 2.61 ppm (integral 2H) was assigned to the methylenic protons at H-7 resulting from reduction of the carbonyl group. Deshielding due to the aromatic protons caused a downfield shift of H-7 compared to the H-8 and H-9. The multiplet at 1.90 ppm (integral 2.0H) was attributed to H-8, and the multiplicity was as expected from the presence of four adjacent methylenic protons (H-7 and H-9). The remaining triplet at 2.33 ppm (integral 2.0H) was attributed to H-9. The aromatic protons remained as two doublets at 7.29 and 7.10 ppm (integrals 1.8 and 1.9H, respectively). The notable differences in the $^{13}$C and DEPT spectra to those of 4-(4′-tert-butylphenyl)-4-oxobutanoic acid (Figure 2.91 and Figure 2.92) are the loss of the carbonyl resonance at 197.6 ppm and the appearance of a methylenic resonance at 34.5 ppm (C-7).
Figure 2.97. $^1$H NMR spectrum of 4-($4'$-tert-butyl phenyl) butanoic acid. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform. Table 2.8 contains details of chemical shifts and integral).

Figure 2.98. $^1$H NMR spectrum of 4-($4'$-tert-butyl phenyl) butanoic acid, expanded region between 1.0 and 3.0 ppm. (Joel EX270MHz HR FT-NMR. Samples dissolved in deuterated chloroform. Table 2.8 contains chemical shifts and integrals).
Figure 2.99. $^{13}$C NMR spectrum of 4-(4'-tert-butylphenyl)butanoic acid. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform. Table 2.8 contains chemical shifts).

Figure 2.100. DEPT NMR spectrum of 4-(4'-tert-butylphenyl)butanoic acid. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform. Table 2.8 contains chemical shifts).
Figure 2.101. $^{13}$C NMR spectrum of 4-(4'-tert-butylphenyl)butanoic acid, expanded region between 25 and 40 ppm. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform. Table 2.8 contains chemical shifts).

Figure 2.102. DEPT NMR spectrum of 4-(4'-tert-butylphenyl)butanoic acid, expanded region between 25 and 40 ppm. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform. Table 2.8 contains chemical shifts).
Table 2.8. Assignment of $^1$H and $^{13}$C resonances in NMR spectra of 4-($^t$er$t$-butylphenyl)butanoic acid.

<table>
<thead>
<tr>
<th>Carbon number</th>
<th>$^1$H chemical shift (Integral)</th>
<th>$^1$C chemical shift (ppm)</th>
</tr>
</thead>
<tbody>
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<td></td>
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<td>$^3$CH$_2$</td>
</tr>
<tr>
<td>1</td>
<td>1.29 (9.2)</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
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<td>-</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>7.29 (1.8)$^1$</td>
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</tr>
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<td>5</td>
<td>7.12 (1.9)$^1$</td>
<td>8.1</td>
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<tr>
<td>7</td>
<td>2.61 (2.0)</td>
<td>8.1</td>
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<tr>
<td>8</td>
<td>1.93 (2.0)</td>
<td>8.1</td>
</tr>
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<td>9</td>
<td>2.33 (2.0)</td>
<td>8.1</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

$^1$ Assignments of H/C4 and H/C5 may be interchanged.

The infrared spectrum of 4-(4-$^t$er$t$-butylphenyl)butanoic acid (Figure 2.93) was very similar to that described for 4-(4-$^t$er$t$-butylphenyl)-4-oxobutanoic acid in section 2.7.1, with the notable difference that the absorbance due to the C=O (1686 cm$^{-1}$) in the keto group was now absent from the spectrum of XI.
2.6.3 Preparation and characterisation of 4-(4'-tert-butylcyclohexyl)butanoic acid

4-(4'-tert-butylphenyl)butanoic acid (XI) was converted into 4-(4'-tert-butylcyclohexyl)butanoic acid (Figure 2.105; XII) via a hydrogenation reaction adapted from Dauben and Hoerger (1951), using platinum oxide as the catalyst and acetic acid as the solvent with a steady stream of hydrogen under pressure (ca. 8 psi; ca. 48 hours). 4-(4'-tert-butylcyclohexyl)butanoic acid was obtained in quantitative amounts (ca. 200 mg) and high purity (>96%) as two isomers shown by GC-MS analysis of the TMS ester (Figure 2.106).

![Reaction scheme for hydrogenation of 4-(4'-tert-butylphenyl)butanoic acid (XI) to the surrogate naphthenic acid 4-(4'-tert-butylcyclohexyl)butanoic acid (tert-BCHBA; III).](image)

Figure 2.104. Reaction scheme for hydrogenation of 4-(4'-tert-butylphenyl)butanoic acid (XI) to the surrogate naphthenic acid 4-(4'-tert-butylcyclohexyl)butanoic acid (tert-BCHBA; III).
Mass spectrometry, in conjunction with NMR and IR, confirmed the structure of 4-(4'-tert-butylcyclohexyl)butanoic acid. The mass spectrum (Figure 2.106) of each isomer was identical, each exhibiting a molecular ion of \( m/z \) 298 (as TMS ester) as expected. Both the loss of a methyl group (\( m/z \) 283; \( M^+ - 15 \)) and an ion at \( m/z \) 117 are characteristic of TMS esters of carboxylic acids. The ions observed at \( m/z \) 255 (\( M^+ - 43 \)) and \( m/z \) 239 (\( M^+ - 59 \)) are characteristic of a butanoic TMS ester substituted cyclohexyl moiety and are discussed more fully for \( \textit{iso-BCHBA} \) in section 2.4.3.

![Figure 2.105. Total ion current gas chromatogram of 4-(4'-tert-butylcyclohexyl)butanoic acid isomers (as TMS ester). (GC-MS conditions: Column, HP-1MS 30 m x 0.25 mm id x 0.25 \( \mu \)m film; oven programme; 40 – 300 °C at 10 °C min\(^{-1}\), hold 10 min; injector 250 °C, ion source 280 °C, 70 eV).](image-url)
Complete characterisation of 4-(4'-tert-butylcyclohexyl)butanoic acid by NMR spectroscopy (Figures 2.108 to 2.111) was not possible due to the complexity of the spectra. This was due to the large number of methylene groups in very similar chemical environments in the two diastereomers of XII. Importantly however, it can be seen from the spectra that the aromatic protons and carbons in XI were no longer present following hydrogenation to XII. The singlet at 0.81 ppm (Figure 2.107; integral 9.7H) has been assigned to the methyl protons H-1. An enlarged area of the singlet reveals another singlet slightly upfield; this is consistent with the diastereomers observed by GC-MS (Figure 2.105). It is likely that the observed integral of 9.7 is above the expected 9 because of integration errors associated with there not being baseline separation between the multiplets. The multiplet at 2.35 ppm has been assigned to the methylenic protons H-9 (integral 1.8). The downfield shift relative to the other proton signals is caused by the deshielding effect of the neighbouring carboxyl moiety. The 14 remaining protons form a complex mix of overlapping multiplets with an integral of 12.8.

Figure 2.106. Representative mass spectrum of 4-(4'-tert-butylcyclohexyl)butanoic acid conformers (as TMS ester). (MS conditions: Ion source 250 °C, ionisation energy 70 eV, mass range 50-550 Daltons).
Infrared spectroscopy (Figure 2.111) confirmed the loss of aromaticity (i.e. loss of absorbances at 3032 and 1513 cm$^{-1}$, while all other functional assignments were the same as those observed for 4-(4'-tert-butylphenyl)butanoic acid.
Figure 2.107. $^1$H NMR spectrum of 4-(4'-tert-butylcyclohexyl)butanoic acid. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform).

Figure 2.108. $^1$H NMR spectrum of 4-(4'-tert-butylcyclohexyl)butanoic acid (0.5 and 2.5 ppm). (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform).
Figure 2.109. $^{13}$C NMR spectrum of 4-(4'-tert-butylcyclohexyl)butanoic acid. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform).

Figure 2.110. DEPT NMR spectrum of 4-(4'-tert-butylcyclohexyl)butanoic acid. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform).
2.7 Synthesis of 4-(4'-iso-butylphenyl)-4-methylbutanoic acid

Full experimental details for the synthesis of 4-(4'-iso-butylphenyl)-4-methylbutanoic acid can be found in Chapter 5.

2.7.1 Preparation and characterisation of 4-(4'-iso-butylphenyl)-4-methylbutanoic acid

4-(4'-iso-butylphenyl)-4-methylbutanoic acid (Figure 2.112; XIII) was formed in reasonable yield (21 %) via a Friedel-Crafts acylation of iso-butylbenzene using γ-valerolactone, with aluminium chloride as a catalyst, and tetrachloroethane as the solvent. The GC-MS total ion current chromatogram of the products as TMS esters revealed three major peaks (Figure 2.113). The mass spectra of the three peaks (Figures 2.115 to 2.117) all exhibited a molecular ion of $m/z$ 306, suggesting that all three peaks are isomers of 4-(iso-butylphenyl)-4-methylbutanoic acid. Preparative high pressure liquid chromatography (prep-HPLC; Figure 2.117) was used to separate and collect milligram quantities of each isomer.
Figure 2.112. Reaction scheme for the synthesis of 4-(4'-iso-butylphenyl)-4-methylbutanoic acid (XIII).

Figure 2.113. Total ion current gas chromatogram analysis of products from the synthesis of 4-(iso-butylphenyl)-4-methylbutanoic acid (as TMS esters). (GC-MS conditions: Column, HP-1MS 30 m x 0.25 mm id x 0.25 μm film; oven programme; 40 – 300 °C at 10 °C min⁻¹, hold 10 min; injector 250 °C).
Figure 2.114. Mass spectrum of 4-(iso-butylphenyl)-4-methylbutanoic acid synthesis product Isomer 1 from Figure 2.113 (as TMS ester). (MS conditions: Ion source 250 °C, ionisation energy 70 eV, mass range 50-550 Daltons).

Figure 2.115. Mass spectrum of 4-(iso-butylphenyl)-4-methylbutanoic acid synthesis product Isomer 2 from Figure 2.113 (as TMS ester). (MS conditions: Ion source 250 °C, ionisation energy 70 eV, mass range 50-550 Daltons).
Figure 2.116. Mass spectrum of 4-((iso-butylphenyl)-4-methylbutanoic acid synthesis product Isomer 2 from Figure 2.113 (as TMS ester). (MS conditions: Ion source 250 °C, ionisation energy 70 eV, mass range 50 -550 Daltons).

Figure 2.117. Preparative HPLC TIC showing three 4-((iso-butylocyclohexyl)-methylbutanoic acid isomers with fraction collection times. (HP1100; 150 x 10.0 mm, 5 μm Gemini C18 HPLC column; mobile phase, methanol:H2O:Formic acid; 75:25:0.1; 3 mL min⁻¹).
2.7.2 Characterisation of 4-(iso-butylphenyl)-4-methylbutanoic acid Isomer 1 from Figure 2.113 and Figure 2.117

GC-MS analysis (Figure 2.118) showed the 3.1 mg collected of isomer 1 to be of high purity (>95%). Analysis of isomer 1 by $^1$H, $^{13}$C and DEPT NMR spectroscopy (Figures 2.120-2.123) led to complete assignment of proton and carbon chemical shifts (Table 2.9). The $^1$H NMR spectrum (Figure 2.119) of 4-(iso-butylphenyl)-4-methylbutanoic acid isomer 1 shows two doublets of resonances in very similar chemical environments (0.98 and 0.88 ppm; joint integral 6.9H). The doublets are assigned to the protons of the methyl groups H-1 and H-1'. The differences in chemical environments of H-1 and H-1' are attributed to a restricted rotation as a consequence of hindrance/influence of the carboxylic acid group. The aromatic protons are seen as a doublet (7.05 ppm; integral 1.9H) and a multiplet (7.20 ppm; integral 2.0H) indicating that substitution of the alkanoic moiety had occurred in the ortho position. The expected signal splitting for H-5 and H-8 in an ortho substituted compound would be two doublets in very similar chemical environments as they each have one neighbouring proton and H-6 and H-7 would be represented by triplets in similar chemical environments as they each have two neighbouring protons. Ortho substitution of the alkanoic moiety results in the close proximity of the isopropyl and carboxylic acid moieties causing steric hindrance and restricted rotation of the protons of the methyl groups H-1 and H-1'. The $^1$H spectrum is further characterised by a doublet at 1.22 ppm (integral 3.0H) attributed to H-11, with the signal splitting caused by a single neighbouring proton (H-10). Two overlapping multiplets at 1.77 and 1.89 ppm (joint integral 3.1H) are attributed to H-2 and H-12, respectively. The similar chemical shift is a result of equal shielding of the aromatic protons. The multiplet at 2.15 ppm (integral 2.1H) is attributed to H-13, with a relative downfield shift caused by deshielding effect of the carboxyl group. The diastereotopic resonances at 2.59 and 2.41 ppm are assigned to H-3. The expected doublet signal splitting is seen as four diastereotopic doublets (integral 1.9H) caused by the restricted rotation of H-1 and H-1'}. This was confirmed by decoupling
experiments where the irradiation of H-2 modified the four diasterotopic doublets to 4 diasterotopic singlets. Decoupling experiments were also used to confirm the assignments of H-1, H-2 and H10-H13. H-10 is seen as a multiplet at 3.09 ppm (integral 1.0H).

The $^{13}$C NMR spectrum (Figure 2.121) of 4-(iso-butylyphenyl)-4-methylbutanoic acid isomer 1 shows two intense resonances at 21.45 and 21.40 ppm which remain above the baseline in the DEPT spectrum (Figure 2.122) indicating that they are either due to a tertiary carbon or to methyl carbons. Thus, these resonances have been assigned to the methyl groups C-1 and C-1' of the isopropyl moiety. The resonance at 21.6 ppm also remains above the baseline in the DEPT spectrum and has been assigned to C-11. Two other resonances in the up-field region remaining above the baseline in the DEPT spectrum at 30.0 and 33.0 ppm assigned to C-2 and C-10, respectively. The resonance at 42.0 ppm is below the baseline in the DEPT spectrum and due to its relative downfield shift has been assigned to C-3, which is deshielded to the aromatic carbons. The remaining two resonances below the baseline in the DEPT spectrum at 33.0 and 31.9 ppm have been assigned to C-13 and C-12, respectively. Of the five resonances seen in the aromatic region, the two at 144.6 and 138.8 ppm are not present on the DEPT spectrum, and thus are assigned to the quaternary carbons of C-4 and C-9. The intense resonance at 125.1 ppm is assigned to C-6 and C-7 as they are in very similar chemical environments, while the less intense resonances at 130.4 and 126.2 ppm are assigned to C-5 and C-8, although the order of assignment is not known.

The characterisation of isomer 1 by $^1$H, $^{13}$C and DEPT NMR spectroscopy has confirmed that the chemical is 4-(2'-iso-butylyphenyl)-4-methylbutanoic acid.
Figure 2.118. Total ion current gas chromatogram analysis of 4-(2'-iso-butylphenyl)-4-methylbutanoic acid (Isomer 1 from Figure 2.113 and Figure 2.117; as TMS ester). (GC-MS conditions: Column, HP-1MS 30 m x 0.25 mm id x 0.25 μm film; oven programme; 40 – 300 °C at 10 °C min\(^{-1}\), hold 10 min; injector 250 °C).

Figure 2.119. \(^1\)H NMR spectrum of 4-(2'-iso-butylphenyl)-4-methylbutanoic acid (Isomer 1 from Figure 2.113 and Figure 2.117). (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated methanol). Assignments shown in Table 2.9.
Figure 2.120. $^1$H NMR spectrum of 4-(2'-iso-butylyphenyl)-4-methylbutanoic acid (Isomer 1 from Figure 2.113 and Figure 2.117; 0.5 – 3.5 ppm). (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated methanol). Assignments shown in Table 2.9.

Figure 2.121. $^{13}$C NMR spectra of 4-(2'-iso-butylyphenyl)-4-methylbutanoic acid (Isomer 1 from Figure 2.113 and Figure 2.117). (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated methanol). Assignments shown in Table 2.9.
Figure 2.122. DEPT NMR spectra of 4-(2'-iso-butylphenyl)-4-methylbutanoic acid (Isomer 1 from Figure 2.113 and Figure 2.117). (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated methanol). Assignments shown in Table 2.9.
Table 2.9. Assignment for $^1$H and $^{13}$C resonances in NMR spectra for 4-(2'-iso-butylphenyl)-4-methylbutanoic acid (Isomer 1 from Figure 2.113 and Figure 2.117).

<table>
<thead>
<tr>
<th>Carbon number</th>
<th>$^1$H chemical shift (Integral)</th>
<th>J (Hz)</th>
<th>$^{13}$C chemical shift (ppm)</th>
</tr>
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<tbody>
<tr>
<td>1/1'</td>
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<td>5.4</td>
<td>CH$_3$ 21.4/21.5$^1$</td>
</tr>
<tr>
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<td>1.77 (3.1)$^2$</td>
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</tr>
<tr>
<td>3</td>
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<td>8.1</td>
<td>42.0</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>-</td>
<td>138.8$^3$</td>
</tr>
<tr>
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<td>7.05 (1.9)</td>
<td>8.1</td>
<td>126.1$^4$</td>
</tr>
<tr>
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<td>125.1</td>
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<tr>
<td>7</td>
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<td>8.1</td>
<td>125.1</td>
</tr>
<tr>
<td>8</td>
<td>7.05 (1.9)</td>
<td>8.1</td>
<td>130.3$^4$</td>
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<td>-</td>
<td>144.6$^3$</td>
</tr>
<tr>
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<td>3.09 (1.0)</td>
<td>9.1</td>
<td>33.0</td>
</tr>
<tr>
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<tr>
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<td>5.4</td>
<td>31.9</td>
</tr>
<tr>
<td>13</td>
<td>2.15 (2.1)</td>
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<td>33.0</td>
</tr>
<tr>
<td>14</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

$^1$ Signal splitting occurred due to the restricted rotation of 1 and 1' as a consequence of hindrance/influence from the carboxylic acid group.

$^2$ Signals from the protons at H-2 and H-12 are seen as overlapping multiplets due to similar chemical environments therefore they have been integrated together.

$^3$ Signals from C-4 and C-9 may be interchanged.

$^4$ Signals from C-5 and C-8 may be interchanged.

2.7.3 Characterisation of 4-(iso-butylphenyl)-4-methylbutanoic acid Isomer 2 from Figure 2.113 and Figure 2.117

GC-MS analysis (Figure 2.123) showed the 7.0 mg collected of isomer 2 to be of high purity (>95%). Analysis of Isomer 2 by $^1$H, $^{13}$C and DEPT NMR spectroscopy (Figures to 2.124 – 2.127) led to complete assignment of proton and carbon chemical shifts (Table 2.10). The $^1$H NMR spectrum (Figure 2.124) of 4-(iso-butylphenyl)-4-methylbutanoic acid isomer 2 was characterised by an intense doublet (integral 6.6H) of resonances at 0.88 ppm assigned to the six protons of the two methyl groups (H-1). The signal splitting (doublet)
was attributed to the effect of an adjacent single neighbouring proton (H-2). This feature is typical of $^1$H NMR spectra of compounds containing the isopropyl ($\text{CH(} \text{CH}_3 \text{)}_2$) moiety and thus helps to confirm the presence of the iso-butyl side chain. Another doublet at 1.24 ppm (integral 3.0H) was attributed to the 3 protons of H-11. Once again the signal splitting (doublet) was attributed to the effect of an adjacent single neighbouring proton (H-10). A multiplet at 1.86 (integral 3.1H) was attributed to the 3 magnetically similar protons in H-2 and H-12. The triplet at 2.12 ppm (integral 2.1H) was due to H-13, and the multiplicity was expected from the presence of two adjacent methylenic protons (H-12). The doublet at 2.44 ppm (integral 2.3) was attributed to H-3; the signal splitting was expected from the single adjacent proton. The multiplet at 2.70 ppm (integral 1.2H) was attributed to H-10, with the multiplicity caused by the neighbouring methyl (H-11) and methylenic protons (H-12). The aromatic protons are seen as a triplet (7.18 ppm; integral 1.0H) and a multiplet (6.97 ppm; integral 2.8H). The triplet with an integral of 1H indicates that only one proton has two adjacent protons, thus confirming that the substitution has occurred in the meta position and the triplet is assigned to H-6. The multiplet at 6.97 ppm is attributed to the protons H-5, H-7 and H-9 and the multiplicity is as expected from two magnetically similar doublets and a singlet arising from H-5, H-7 and H-9, respectively.

The $^{13}$C NMR spectrum of 4-(iso-butylphenyl)-4-methylbutanoic acid isomer 2 (Figure 2.126) shows an intense resonance at 21.4 ppm which remains above the baseline in the DEPT spectrum (Figure 2.126) indicating that it is either a tertiary or a methyl carbon. The DEPT spectrum shows two other resonances (30.2 and 39.3 ppm) that remain above the baseline in the 0 – 50 ppm region which are approximately a third of the intensity of the 21,4 ppm resonance. Thus, the resonance at 21.4 ppm has been assigned to the effects of the methyl carbons H-1 and H-11. The remaining two resonances above the baseline have been assigned to C-2 (30.2 ppm) and C-10 (39.3 ppm) with the deshielding effect of the aromatic carbons causing the relative downfield shift of C-10 compared to C-2. The
resonance below the baseline in the DEPT spectrum at 45.2 ppm has been assigned to C-3. Again the deshielding effect of the aromatic carbons caused a downfield shift of C-3 compared to C-12 and C-13 (which are also below the baseline 32.1 and 33.3 ppm, respectively). The four resonances between 120 – 130 ppm are attributed to aromatic carbons C-5, C-6, C-7 and C-9. Their presence on the DEPT spectrum confirms that they are not quaternary. The quaternary aromatic carbons, C-4 and C-8, are not observed in the $^{13}$C spectrum as the sample was analysed at a very low concentration. The same is true for the carbonyl carbon (C-14).

The characterisation of isomer 2 by $^1$H, $^{13}$C and DEPT NMR spectroscopy has confirmed it to be 4-(3’-iso-butylphenyl)-4-methylbutanoic acid.

![Figure 2.123. Total ion current gas chromatogram analysis of 4-(3’-iso-butylphenyl)-4-methylbutanoic acid (Isomer 2 from Figure 2.113 and Figure 2.117; as TMS ester). (GC-MS conditions: Column, HP-1MS 30 m x 0.25 mm id x 0.25 μm film; oven programme; 40 – 300 °C at 10 °C min$^{-1}$, hold 10 min; injector 250 °C).](image-url)
Figure 2.124. $^1$H NMR spectrum of 4-(3’-iso-butylphenyl)-4-methylbutanoic acid (Isomer 2 from Figure 2.113 and Figure 2.117). (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated methanol). Assignments shown in Table 2.10.

Figure 2.125. $^1$H NMR spectrum of 4-(3’-iso-butylphenyl)-4-methylbutanoic acid (Isomer 2 from Figure 2.113 and Figure 2.117; expanded region 0.5 – 3.0 ppm). (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated methanol). Assignments shown in Table 2.10.
Figure 2.126. $^{13}$C NMR spectra of 4-(3'-iso-butylphenyl)-4-methylbutanoic acid (Isomer 2 from Figure 2.113 and Figure 2.117). (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated methanol). Assignments shown in Table 2.10.

Figure 2.127. DEPT NMR spectrum of 4-(3'-iso-butylphenyl)-4-methylbutanoic acid (Isomer 2 from Figure 2.113 and Figure 2.117). (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated methanol). Assignments shown in Table 2.10.
Table 2.10. Assignment for $^1$H and $^{13}$C resonances in NMR spectra for 4-(3'-iso-butylphenyl)-4-methylbutanoic acid (Isomer 2 from Figure 2.113 and Figure 2.117).

<table>
<thead>
<tr>
<th>Carbon number</th>
<th>$^1$H chemical shift (Integral)</th>
<th>$^1$H chemical shift (Hz)</th>
<th>$^{13}$C chemical shift (ppm)</th>
</tr>
</thead>
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<td>-</td>
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<td>6.97 (2.8)</td>
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</tr>
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<td>14</td>
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<td>-</td>
</tr>
</tbody>
</table>

1 Signals from the protons at H-2 and H-12 are seen as overlapping multiplets due to similar chemical environments therefore they have been integrated as one.

2 Signals from the protons at H-5, H-7 and H-9 are seen as overlapping multiplets due to similar chemical environments therefore they have been integrated as one.

3 Assignments for C5, C6, C-7 and C-9 may be interchanged.

4 Signals not seen due to low sample mass.

2.7.4 Characterisation of 4-(iso-butylphenyl)-4-methylbutanoic acid Isomer 3 from Figure 2.113 and Figure 2.117

GC-MS analysis (Figure 2.128) showed the 4.1 mg collected of Isomer 3 to be of high purity (>98%). Analysis of isomer 1 by $^1$H, $^{13}$C and DEPT NMR spectroscopy (Figures to 2.129 – 2.132) led to complete assignment of proton and carbon chemical shifts (Table 2.11). The $^1$H NMR spectrum (Figure 2.129) of 4-(iso-butylphenyl)-4-methylbutanoic acid Isomer 3 was characterised by an intense doublet (integral 5.9H) of resonances at 0.87 ppm.
assigned to the six protons of the two methyl groups (H-1). The signal splitting (doublet) was attributed to the effect of an adjacent single neighbouring proton (H-2). This feature is typical of $^1$H NMR spectra containing the isopropyl (–CH(CH$_3$)$_2$) moiety and thus helps to confirm the presence of the iso-butyl side chain. Another doublet at 1.23 ppm (integral 3.0H) was attributed to the three protons of H-9. Again the signal splitting (doublet) was attributed to the effect of an adjacent single neighbouring proton (H-8). A multiplet at 1.85 (integral 3.1) was attributed to the three magnetically similar protons in H-2 and H-10. The triplet at 2.12 ppm (integral 1.9H) was due to H-11, and the multiplicity was expected from the presence of two adjacent methylenic protons (H-10). The doublet at 2.42 ppm (integral 2.0H) was attributed to H-3; the signal splitting was expected from the single adjacent proton (H-2). The multiplet at 2.69 ppm (integral 1.0H) was attributed to H-8, with the multiplicity caused by the neighbouring methyl protons (H-9) and methylenic protons (H-10). The proton spectrum also shows that the aromatic protons are magnetically equivalent, observed as a singlet (7.07 ppm; integral 4.0H), indicating that the compound is para disubstituted.

The $^{13}$C NMR spectrum of 4-(iso-butylphenyl)-4-methylbutanoic acid isomer 2 (Figure 2.131) shows an intense resonance at 21.4 ppm which remains above the baseline in the DEPT spectrum (Figure 2.132) indicating that it is due to either a tertiary or a methyl carbon. The DEPT spectrum shows two other resonances (30.2 and 39.0 ppm) that remain above the baseline in the 0–50 ppm region which are approximately a third of the intensity. Thus, the resonance at 21.4 ppm has been assigned to the methyl carbons H-1 and H-9. The remaining two resonances above the baseline have been assigned to C-2 (30.2 ppm) and C-10 (39.0 ppm) with the deshielding effect on C-8 from the aromatic carbons causing the relative downfield shift compared to C-2. The resonance below the baseline in the DEPT spectrum at 44.8 ppm has been assigned C-3. Once again the deshielding effect to the aromatic carbons caused a downfield shift compared to C-11 and

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C-10 which are also below the baseline (32.0 and 33.3 ppm, respectively). The aromatic carbons show resonances between 125 and 150 ppm, of which the two small resonances at 143.7 and 139.63 ppm are not present on the DEPT spectrum and so are due to quaternary carbons (C-4 and C-7). The four remaining carbons of the aromatic ring are seen as two resonances (128.9 and 126.3 ppm), which provides further evidence that the compound is para disubstituted.

The characterisation of isomer 3 by \(^1\)H, \(^{13}\)C and DEPT NMR spectroscopy has confirmed it to be 4-(4'-iso-butylphenyl)-4-methylbutanoic acid.

![Figure 2.128. Total ion current gas chromatogram analysis of 4-(4'-iso-butylphenyl)-4-methylbutanoic acid (Isomer 3 from Figure 2.113 and Figure 2.117; as TMS ester). (GC-MS conditions: Column, HP-1MS 30 m x 0.25 mm id x 0.25 μm film; oven programme; 40 – 300 °C at 10 °C min\(^{-1}\), hold 10 min; injector 250 °C).]
Figure 2.129. $^1$H NMR spectrum of 4-(4'-iso-butyrylphenyl)-4-methylbutanoic acid (Isomer 3 from Figure 2.113 and Figure 2.117). (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated methanol). Assignments shown in Table 2.11.

Figure 2.130. $^1$H NMR spectrum of 4-(4'-iso-butyrylphenyl)-4-methylbutanoic acid (Isomer 3 from Figure 2.113 and Figure 2.117; expanded region 0.5 – 3.0 ppm). (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated methanol). Assignments shown in Table 2.11.
Figure 2.131. $^{13}$C NMR spectrum of 4-(4'-iso-butylphenyl)-4-methylbutanoic acid (Isomer 3 from Figure 2.113 and Figure 2.117). (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated methanol). Assignments shown in Table 2.11.

Figure 2.132. DEPT NMR spectrum of 4-(4'-iso-butylphenyl)-4-methylbutanoic acid (Isomer 3 from Figure 2.113 and Figure 2.117). (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated methanol). Assignments shown in Table 2.11.
Table 2.11. Assignment for $^1$H and $^{13}$C resonances in NMR spectra for 4-(4'-iso-butylphenyl)-4-methylbutanoic acid Isomer 3.

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<th>Carbon number</th>
<th>$^1$H chemical shift (Integral)</th>
<th>$^1$H J (Hz)</th>
<th>$^{13}$C chemical shift (ppm)</th>
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<td>128.9$^4$</td>
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<td>126.4$^4$</td>
</tr>
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<tr>
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<td>2.12 (1.9)</td>
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<td>33.3</td>
</tr>
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</tbody>
</table>

1. Signals from the protons at H-2 and H-10 are seen as overlapping multiplets due to similar chemical environments. Therefore they have been integrated together.

2. Protons for H-5 and H-6 are magnetically equivalent.

3. Assignments for C4 and C7 may be interchanged.

4. Assignments for C5 and C6 may be interchanged.

5. Signals not seen due to low sample amount.

2.7.5 Hydrogenation of 4-(4'-iso-butylphenyl)-4-methylbutanoic acid

4-(4'-iso-butylphenyl)-4-methylbutanoic acid (XIII) was converted into 4-(4'-isobutylcyclohexyl)-4-methylbutanoic acid (iso-BCHMBA; Figure 2.133; XIV) via a hydrogenation reaction adapted from Dauben and Hoerger (1951), using platinum oxide as the catalyst and acetic acid as the solvent with a steady stream of hydrogen under pressure (ca. 8 psi). 4-(4'-iso-butylcyclohexyl)butanoic acid was obtained in quantitative amounts (ca. 4 mg) and high purity (>96 %) as two isomers shown by GC-MS analysis of the TMS esters (Figure 2.134).
Mass spectrometry was used to confirm the structure of 4-(4'-iso-butylcyclohexyl)-4-methylbutanoic acid. The mass spectra (Figure 2.135) of both isomers were identical, each exhibiting a molecular ion of m/z 312 (as TMS esters) as expected. Both the loss of a methyl (m/z 297; M^+ - 15) and a base peak ion of m/z 117 are characteristic of TMS esters of carboxylic acids. The ions observed at m/z 255 (M^+ - 57) and m/z 239 (M^+ - 73) are characteristic of rearrangements of the butanoic TMS ester substituted cyclohexyl moiety and were discussed more fully for iso-BCHBA in section 2.4.3. Insufficient amounts of 4-(4'-iso-butylcyclohexyl)-4-methylbutanoic acid were obtained to carry out NMR spectroscopy. However, it was deemed that the NMR data for the aromatic intermediate and the mass spectral data of the hydrogenated product were sufficient to characterise 4-(4'-iso-butylcyclohexyl)-4-methylbutanoic acid.
Figure 2.134. Total ion current gas chromatogram of 4-(4′-iso-butylcyclohexyl)-4-methylbutanoic acid (as TMS ester). (GC-MS conditions: Column, HP-1MS 30 m x 0.25 mm id x 0.25 µm film; oven programme; 40 – 300 °C at 10 °C min⁻¹, hold 10 min; injector 250 °C, ion source 280 °C, 70 eV).

Figure 2.135. Representative mass spectrum of 4-(4′-iso-butylcyclohexyl)-4-methylbutanoic acid conformers (as TMS ester). (MS conditions: Ion source 250 °C, ionisation energy 70 eV, mass range 50 -550 Daltons).
2.8 Conclusions

4-(4'-iso-butyryclohexyl)butanoic acid, 4-(4'-n-butyryclohexyl)butanoic acid, 4-(4'-sec-butyryclohexyl)butanoic acid and 4-(4'-tert-butyryclohexyl)butanoic acid were all synthesised in good yield (~200 mg) and purity (>94% by GC). In addition, 4-(4'-iso-butyryclohexyl)-4-methylbutanoic acid was synthesised (ca. 4 mg; >96% pure by GC). Intermediates at each stage of the synthetic pathway were characterised by GC-MS, and by IR and NMR spectroscopy. These compounds are thought to be good surrogates of the compounds present in complex mixtures of natural NAs.

NMR spectroscopy is the only technique that provides unequivocal stereochemical confirmation of structure at a molecular level. However, the complete characterisation of the NA surrogate diastereomer products by NMR was not possible, and characterisation was achieved through characterisation of the intermediate precursors in the synthetic route and by use of other spectrometric techniques (IR and GC-MS) to characterise the final products. This difficulty in characterising known surrogate diastereomer NAs arises from the large number of similar atoms in similar chemical environments (e.g. methylenic groups) and in turn explains the difficulties previously encountered in characterisation of unknown complex mixtures of NAs even by the most sophisticated techniques.

Such NA surrogates are unavailable from commercial sources and were synthesised in quantities large enough to allow thorough structural characterisation, and pure enough for subsequent toxicological (Chapter 3), biodegradation (Chapter 3) and analytical method development studies (Chapter 4) to be made. These should increase our understanding of the fate and effects of NAs in the environment and represent a significant advance on previous studies.
Chapter 3  Toxicity and biodegradation studies of surrogate NAs

3.1  Introduction

3.1.1 Toxicity of naphthenic acids

The toxicity of NAs has been well documented (e.g. Clemente and Fedorak, 2005). They are believed to be amongst the most toxic components of oil refinery effluents and oil sands tailing waters where they are present in concentrations ranging between 20-120 mg L⁻¹ and in quantities exceeding 600 x 10⁶ m³ at just one extraction site (Syncrude Mildred Lake; Scott et al., 2005; Clemente and Fedorack, 2005). The toxicity is often associated with their surfactant characteristics (MacKinnon and Boerger, 1986; Rogers et al., 2002a; Rogers et al., 2002b), which allow NAs to more easily penetrate cell walls (Quagraine et al., 2005b). Previous studies using mixtures of NAs from commercial and environmental sources have shown toxic effects to plants, bacteria, zooplankton, fish and rats. A summary of toxicity studies with naphthenic acids is presented in Table 3.1. These studies demonstrate the real concern regarding tailing waters as the lethal dose concentrations for bacteria (LC₅₀ 5 mg L⁻¹; Clemente et al., 2004), zooplankton (tolerance of 0.15 mg L⁻¹; Dokholyan and Magomedov, 1984) and fish (100 % mortality 6.8 mg L⁻¹; Nero et al., 2005) lie well below those present.
Table 3.1. Summary of toxicity studies with naphthenic acids. Adapted from Clemente and Fedorak (2005).

<table>
<thead>
<tr>
<th>Organism</th>
<th>Reference</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria</td>
<td>Holowenko et al., (2002)</td>
<td>Microtox was used to monitor the toxicity of different aged oil sands tailings ponds.</td>
</tr>
<tr>
<td>Bacteria</td>
<td>Clemente et al., (2004)</td>
<td>Microtox was used to monitor an aerobic biodegradation study. The study demonstrated that biodegradation of NAs reduced the toxicity of culture supernatants. IC50 values for NA preparations was ~5 mg L⁻¹.</td>
</tr>
<tr>
<td>Aspen</td>
<td>Kamaluddin and Zwiazek (2002)</td>
<td>Growth, chlorophyll production, photosynthetic rates, root water flow, and root respiration decreased in the presence of NAs in the concentrations of 75 to 300 mg L⁻¹ of growth medium.</td>
</tr>
<tr>
<td>Zooplankton</td>
<td>Dokholyan and Magomedov (1984)</td>
<td>This species tolerated naphthenic acids concentrations up to only 0.15 mg L⁻¹. Thus, 0.15 mg L⁻¹ was suggested as the maximum allowable concentration of naphthenic acids in waters.</td>
</tr>
<tr>
<td>Fish</td>
<td>Dokholyan and Magomedov (1984)</td>
<td>Studied acute toxicity by exposing fish species to 12 - 100 mg L⁻¹ NAs for 10 d. 50% mortality was observed with 2-month-old chum salmon exposed to 25 mg L⁻¹ NAs; with 2-month-old kutum, roach fingerling, and 2-year-old sturgeon exposed to 50 mg L⁻¹ NAs, and with 2-year-old roach and Caspian round goby exposed to 75 mg L⁻¹ NAs.</td>
</tr>
<tr>
<td>Fish</td>
<td>Nero et al., (2005)</td>
<td>Studied effects of salinity on NA toxicity to yellow perch. 6.8 3.6 mg L⁻¹ environmental and commercial NAs, respectively, caused 100% mortality, regardless of salt addition.</td>
</tr>
<tr>
<td>Rats</td>
<td>Rogers et al., (2000b)</td>
<td>To determine the acute toxicity of naphthenic acids, rats were fed a single dose of 3, 30, or 300 mg kg⁻¹ NAs. Toxic effects were observed in animals that received the highest dose. These included organ damage and increased organ weight. In particular, females had heavier spleen and ovaries compared to controls. Males had heavier hearts and testes compared to controls. Liver and heart damage were more prevalent in females, while males experienced more brain haemorrhages. To test the effects of prolonged, subchronic intake of naphthenic acids, female rats were given 0.6, 6, or 60 mg kg⁻¹ NAs, 5 d a week, for 90 d. Twenty five percent of rats receiving 60 mg kg⁻¹ and 17% of rats receiving 6 mg kg⁻¹ experienced severe seizures. The most pronounced increase in organ weight was found in the 60 mg kg⁻¹ group, whose livers were 36% heavier than the controls. Their kidneys and brain were also significantly heavier.</td>
</tr>
</tbody>
</table>

3.1.2 Biodegradation of naphthenic acids

It is estimated that at the current rate of oil sand processing there will be 1 billion m³ of tailing pond waters requiring reclamation by 2025 (Rogers et al., 2002b; Herman et al., 1994). Bioremediation is one of the primary reclamation techniques and this has led to huge interest in biodegradation of NAs over the past decade. A recent review of the biodegradation of NAs suggests that previous NA biodegradation studies fit into three categories, where the substrates have been either surrogate NAs, commercially available
NAAs or those isolated from environmental samples. Studies using surrogate NAAs have been limited by the lack of availability of commercial compounds with the accepted C_nH_{2n+2}O_2 formula (Clemente and Fedorack et al., 2005). Surrogate NAAs that have been proposed include cyclohexylcarboxylic acid, cyclohexanepentanoic acid, 2-methylcyclohexanecarboxylic acid (Herman et al., 1994), cyclohexanepropanoic acid, cyclohexylbutanoic acid, cyclohexylpentanoic acid (Holowenko et al., 2001), 2-methylcyclohexane carboxylic acid, trans-4-pentylcyclohexanecarboxylic acid (Clemente et al., 2003; Herman et al., 1994) and decahydronaphthoic acid (Holowenko et al., 2001; Lai et al., 1996). Previous biodegradation studies using surrogate NAAs are summarised in Table 3.2, which also indicates the structures of the surrogates, the sources of the cultures used and gives an indication of the rate of biodegradation observed, where applicable.
Previous studies have shown that substituted cyclohexanecarboxylic acids are less susceptible to aerobic biodegradation than cyclohexanecarboxylic acid (Herman et al., 1993). Also, alkylethanolcarboxylic acids containing an odd number of carbon atoms in the alkyl chain between the carbonyl group and the ring were far less resistant than those containing an even number of carbon atoms (Headley and McMartin, 2004).

Another recent review by Quagraine et al. (2005), comprehensively lists the microorganisms used for NA biodegradation studies along with the surrogate NAs used and the main products observed. The authors concluded that a wide variety of bacteria
have the ability to metabolise NAs and that the more recalcitrant NAs are those with the following properties: alkyl substituted aliphatic chains; ternary substitution at positions other than the β-position to the carboxylic acid of the main carbon chain; methyl substitution on the ring of the cyclic acids and cis-isomerism of such compounds. The need to have mixed cultures of bacteria to degrade recalcitrant NAs was emphasised as often the mixed cultures succeeded in degrading NAs where single strains failed (Quagraine et al., 2005b).

A third recent review on NA biodegradation concluded that the recalcitrant nature of NAs in aquatic systems appears to be related to the individual structures of NAs, in much the same way that toxicity is structurally dependent (Headley and McMartin, 2004).

Commercially available NAs and those extracted from oil sands tailings waste water have been degraded by aerobic and anaerobic indigenous microbial communities given appropriate nutrient conditions (Herman et al., 1993; Herman et al., 1994; Lai et al., 1996; Clemente 2004; Clemente et al., 2004; Holowenko et al., 2001). These studies have shown that NAs with 'lower' molecular weights and 'fewer' rings (carbon numbers <14 and z numbers 0 and -2) are most susceptible to biodegradation.

Watson et al., (2002) demonstrated in laboratory studies that during aerobic biodegradation of crude oil, significant amounts of carboxylic acids were produced. The rapidly produced medium molecular weight (C_{10} - C_{20}) carboxylic acids were removed after extensive biodegradation leaving increased concentrations of higher molecular weight (>C_{20}) branched and cyclic carboxylic acids which appeared as an unresolved complex mixture (UCM) when analysed by gas chromatography (GC).

Aitken et al., (2004) reported that isolation of metabolites suggests that anaerobic hydrocarbon degradation is a common process in biodegraded subsurface oil reservoirs. They suggest, for example, that decahydro-2-naphthoic acid (a simple bicyclic NA) is a possible dead-end metabolite formed from naphthalene. They conclude that oil
biodegradation in most reservoirs must have been anaerobic in nature at some point so that anaerobic hydrocarbon degradation is the prevailing mechanism for deep subsurface petroleum biodegradation.

The value of bioremediation is emphasised by studies in which biodegradation has resulted in a reduction of the toxicity of culture supernatants (Herman et al., 1994; Clemente et al., 2004). However, at least one study reported no decrease in toxicity of NAs following biodegradation (Lai et al., 1996).

3.1.3 Current aims

In the present study the toxicity of four synthetic surrogate NAs, 4-(4'-n-butylcyclohexyl)butanoic acid (n-BCHBA, Figure 3.1), 4-(4'-iso-butylcyclohexyl)butanoic acid (iso-BCHBA, Figure 3.1), 4-(4'-sec-butylcyclohexyl)butanoic acid (sec-BCHBA, Figure 3.1) and 4-(4'-tert-butylcyclohexyl)butanoic acid (tert-BCHBA, Figure 3.1) was examined in an oyster embryo assay which assesses the development of the embryos after 24h inoculation. Toxicities were compared to those of natural NAs, so far as the different methods allowed. The aim here was to help establish whether the synthetic surrogates were comparable to real NAs and thus could be considered good model NAs. The second stage of the study was to access the biodegradation rates of the unique surrogates using mixed bacterial cultures isolated from local environmental sites in order to determine the potential for bioremediation. Concentrations of the substrates and of metabolites were monitored by gas chromatography (GC) and identities confirmed by GC-MS. Although not reported here, the changes in the bacterial populations during the degradation experiments were also monitored by application of preliminary molecular biological methods such as polymerase chain reaction (PCR) amplification of bacterial DNA, denaturing gradient gel electrophoresis (DGGE) of the DNA and amino acid sequencing of selected DNAs (personal communication; Max Frenzel, University of Exeter; Smith et al., 2006 submitted).
3.2 Results and discussion

3.2.1 Acute oyster embryo larval development test: Initial screening study

The Pacific oyster, *Crassostrea gigas*, was chosen to be used in the embryo development test as a measure of acute toxicity. This assay is an accepted regulatory test used by the UK Environment Agency and an American Standard Test Method (ASTM E 724). The test yields information concerning the acute effects of a short term exposure of organisms to a test material under specific environmental conditions. The results of these acute tests are often considered to be a good indication of the ‘acceptability’ of pollutant concentrations, and are useful for studying biological availability of, and structural activity relationships between, test materials (ASTM E 724). The fertilised eggs are inoculated with the substrate and left for 24h. After 24h the fertilised eggs/developed embryos are fixed with formalin and observed under a microscope. Healthy embryos are a perfect capital ‘D’ shape (Figure 3.2a) and are classed as normal. Unhealthy embryos are deformed from the normal ‘D’ shape (Figure 3.2b) and are classed as abnormal. Fertilised eggs that are subjected to
severe toxins do not progress pass the trochophore stage (Figure 3.2c) and are also classed as abnormal. The results of the assay are presented as a percentage of normal embryos to those which are abnormal. The results of percentage normal embryos for the surrogate NAs at various concentrations can be compared to each other and to the controls.

Figure 3.3 shows the results for the initial embryo larval development test. This experiment sought to allow a comparison of the toxicities of monocyclic, bicyclic and tetracyclic acids. The controls showed that 70 % of the embryos were normally developed in just sea water while 74 % were normal in the solvent control, both of which are considered acceptable percentages, and there was 100 % abnormality in the positive control (Zinc 0.1 mg L⁻¹). It can be seen that at the lowest concentration there was little difference in toxicity between sec-BCHBA and DHNA (p = 0.57), but significant (P = <0.05) difference between each of these and cholanic acid (Figure 3.3). At the lowest concentration (0.001 mg L⁻¹) just over 50 % of the embryos were normally developed for sec-BCHBA and DHNA while only 12 % were normal with CA. All of the concentration points were significantly different for each compound. The Bakers Formalin used from stock did not totally fix the embryos which meant that some embryos did not close up completely, causing a small proportion of the embryos to be classed as false negatives (i.e. classed as abnormal when in fact they were normal). In order to address this issue, the same concentration range was chosen for the full study and fresh Bakers Formalin was prepared.

Figure 3.2. Light micrographs of fertilised oyster embryos used in the toxicity assay of surrogate NAs (a) normal embryo, (b) abnormal embryo and (c) trochophore.
Figure 3.3. Percentage of normal embryos after 24 hours exposure to (a) Control inocula, (b) sec-BCHBA, (c) DHNA and (d) Cholanic acid. Error bars represent the range of duplicates within one assay (experimental details described on pages 224-227).

3.2.2 Acute oyster embryo larval development test: Detailed study

Figure 3.4 shows the percentage of normal embryos for sea water controls, solvent controls and positive controls while Figure 3.5 shows the results for the four synthetic surrogate
NAs at a range of concentrations (0.001 to 1.0 mg L\(^{-1}\)). It can be seen from the histograms that not all of the lower concentrations were significantly different for individual surrogate NAs. 75% of the embryos were normal in the sea water control while 84% were normal in the solvent control, both of which were considered acceptable percentages. Although the difference is not statistically different (\(P > 0.05\)), the solvent controls are expected to have a slightly higher percentage of normal embryos due to a small homeostatic effect. No embryos were normal shape in the zinc (0.1 mg L\(^{-1}\)) positive control, most not progressing past the trochophore stage. Figure 3.7 shows a scatter plot of the percentage normal embryos against concentration for the four surrogates. It can be seen that tert-BCHBA was the least toxic, sec-BCHBA and iso-BCHBA were similarly toxic to each other but more toxic than tert-BCHBA, whilst n-BCHBA was the most toxic. EC50 values are presented in Table 3.3.

![Graph showing percentage of normal embryos against concentration for control inocula.](image)

**Figure 3.4.** Percentage of normal embryos after 24 hours exposure to control inocula. Error bars represent ± \(\sigma\) calculated from triplicates within one assay (experimental details described on pages 224-227).
Figure 3.5. Percentage of normal embryos after 24 hours exposure to (a) n-BCHBA, (b) iso-BCHBA, (c) sec-BCHBA and (d) tert-BCHBA. Individual R groups correspond to structure shown in Figure 3.6. Error bars represent ± σ calculated from triplicates within one assay (experimental details described on pages 224-227).
Figure 3.6. General structure of surrogate NAs used in toxicity assays. R groups are as shown in Figure 3.5.

Figure 3.7. Changes in percentage of normal embryos with varying surrogate NA concentrations (trendline is an exponential fit).

Table 3.3. EC50 values for surrogate NAs. Values are taken from the exponential trendline in Figure 3.7.

<table>
<thead>
<tr>
<th>Surrogate</th>
<th>Concentration (mg L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n)-BCHBA</td>
<td>0.11</td>
</tr>
<tr>
<td>(iso)-BCHBA</td>
<td>0.21</td>
</tr>
<tr>
<td>(sec)-BCHBA</td>
<td>0.33</td>
</tr>
<tr>
<td>(tert)-BCHBA</td>
<td>0.49</td>
</tr>
</tbody>
</table>

Abnormal development in the embryos was the result of a non-specific mode of toxic action induced by exposure to the test compounds. \(n\)-BCHBA was the most toxic (EC50 = 0.11 mg L\(^{-1}\)) of the four surrogates tested and \(tert\)-BCHBA the least (EC50 = 0.49 mg L\(^{-1}\)).
while iso-BCHBA and sec-BCHBA were not statistically different from each other (EC50 = 0.21 and 0.33 mg L⁻¹, respectively), although were significantly different to n-BCHBA and tert-BCHBA.

Meyer and Overton both suggested in 1899 (see Donkin, 1994) that narcosis was the result of partition into lipid phases of the cell; a process that could be accurately modelled by the partition ratio between olive oil and water. This is historically the beginning of Quantitative Structure-Activity Relationship (QSAR) studies. The partitioning ratio between olive oil and water is now replaced by the log₁₀ octanol:water partition coefficient (log Kow). More recently, Donkin et al., (1989) showed that a reduction in mussel feeding rate was negatively correlated with log Kow and positively correlated with aqueous solubility, indicating that hydrophobicity has a major influence on toxicity (at least for mussels). The log Kow values for the four surrogate NAs were calculated herein using Kowwin modelling software. Kowwin is one of many models in the Estimation Programme Interface (EPI) Suite developed by the United States Environmental Protection Agency to model physical/chemical properties and environmental fate. The values obtained were 5.72, 5.65, 5.65 and 5.61 for n-BCHBA, iso-BCHBA, sec-BCHBA and tert-BCHBA, respectively. Figure 3.8 shows that toxicity data from the current study exhibit this same trend. This suggests that a QSAR exists between the structures of the NAs and their toxic effects to the oyster embryos, attributed to their aqueous solubility (log Kow coefficient values) and their efficiency in partitioning into lipid membranes. As branching in the butyl chain is the only difference in the surrogate NAs, this branching is responsible for differences in estimated log Kow and differences in toxicity, but likely does not explain the overall toxic effect. Overton also thought the excess toxicity of compounds such as aldehydes could be due to reactivity; this may also be true for the observed toxicity of the carboxylic acids.
Figure 3.8. Relationships between surrogate NA EC50 values and log $K_{ow}$ and water solubilities as predicted by EPI Suite software.
3.2.3 Initial mixed consortium biodegradation screening study

Figure 3.9 shows the results from the initial screening study for the biodegradation of NA surrogates by five different consortia at 25 °C over 35 days, along with data for abiotic controls. The experiment used 1-adamantanecarboxylic acid and \( n \)-eicosane as internal standards so that the data could be adjusted for sample work-up of polar and possible non-polar metabolites, respectively. The aim of this initial screening was to establish which consortia were best adapted to degrading NA surrogates, so that they could then be used in future, more detailed studies. All five consortia degraded >95% of the monocyclic surrogates (\( n \)-BCHBA and sec-BCHBA) while the bicyclic surrogate (decahydro-2-naphthoic acid) was far more resistant, with >75% remaining after 35 days. The viability of each bacterial consortium used was tested at the point of sampling (Section 5.5.3) and bacterial growth (25 °C; 24h) was observed in all samples - demonstrating that lack of biodegradation could not be attributed to non-viable bacteria. The abiotic controls showed no losses, indicating that degradation was attributable to bacterial action. Of the 4 consortia which showed total degradation of \( n \)-BCHBA and sec-BCHBA, the Devonport consortium was chosen for the more detailed study. This culture is so-named because it was collected from the river Tamar flats at Devonport, Plymouth, UK (full details of all cultures are presented in Section 5.5).
Figure 3.9. Initial biodegradation screening percentage recovery of surrogate NAs subjected to a variety of bacterial consortia over 35 days at 25 °C (bars indicate range; n=2).
3.2.4 Detailed mixed culture biodegradation of surrogate NAs using devonport consortium

Figure 3.10 shows the results for the detailed mixed culture biodegradation of surrogate NAs using the Devonport consortium including sampling points throughout the study period (30 days) at days 0, 1, 3, 9, 14, 21 and 30. The proportions of the NA surrogates (n-BCHBA, iso-BCHBA, sec-BCHBA and tert-BCHBA) recovered after exposure to the Devonport consortium are shown as mean values (± σ, n=3). Results for the abiotic controls for each surrogate confirm that losses were due to biotic degradation. Bacterial viability was tested for all samples at the point of sampling, and bacterial growth (25 °C; 24h) was observed in all samples apart from abiotic controls where no growth was observed. The degradation rates for the four surrogate NAs (n-BCHBA, iso-BCHBA, sec-BCHBA and tert-BCHBA) were significantly different from each other over the 30 day incubation period (Figure 3.10). Tert-BCHBA was the most resistant with 98 % recovery after 30 days; in contrast n-BCHBA was the least resistant to biodegradation with <3 % recovered at day 9. Table 2.2 summarises the recovery of the surrogate NAs after 30 days incubation.
Figure 3.10. Percentage recovery of (a) n-BCHBA, (b) iso-BCHBA, (c) sec-BCHBA and (d) tert-BCHBA subjected to biodegradation by the Devonport consortium over 30 days at 25 °C (points indicate mean; error bars represent ± σ, n=3). Abiotic controls shown in circles (mean ± σ, n = 3).
Table 3.4. Percentage recovery of surrogate NAs after 30 days incubation with Devonport bacterial consortium.

<table>
<thead>
<tr>
<th>Surrogate</th>
<th>Structure</th>
<th>Percentage Recovery (Degradation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-BCHBA</td>
<td><img src="image" alt="n-BCHBA Structure" /></td>
<td>2.9 (97.1)</td>
</tr>
<tr>
<td>iso-BCHBA</td>
<td><img src="image" alt="iso-BCHBA Structure" /></td>
<td>22.6 (77.4)</td>
</tr>
<tr>
<td>sec-BCHBA</td>
<td><img src="image" alt="sec-BCHBA Structure" /></td>
<td>53.4 (46.6)</td>
</tr>
<tr>
<td>tert-BCHBA</td>
<td><img src="image" alt="tert-BCHBA Structure" /></td>
<td>97.5 (2.5)</td>
</tr>
</tbody>
</table>

Gas chromatograms (GC-FID) of the four surrogate NAs recovered after biodegradation at day 30 are presented in Figure 2.10. The internal standard, 1-adamantanecarboxylic acid, eluted with a retention time of 16.13 minutes whilst the surrogate NAs ranged in retention times from 19.11 (tert-BCHBA) to 19.82 min (n-BCHBA). The major metabolites generated during biodegradation had shorter retention times than the surrogates, ranging from 16.70 (tert-BCHBA) to 17.31 minutes (n-BCHBA).

Figure 3.12 and Figure 3.13 show the GC-FID chromatograms of n-BCHBA and iso-BCHBA, respectively, at each sampling point. The chromatograms show not only the degradation of the surrogates but also the production of metabolites. Similar results were also observed for sec-BCHBA and tert-BCHBA surrogates. Identification of the major metabolites produced during biodegradation of the surrogate NAs was attempted using GC-MS and is discussed later (page 154).

It can be seen from Figure 3.12 and Figure 3.13 that the individual diastereomers degrade at different rates. As the isomers are not baseline resolved it is hard to accurately quantify...
the difference in degradation rates. However it is clear that in each case the later eluting, less abundant isomer degrades faster than the earlier eluting more abundant isomer. A study by Headley et al., (2002) suggested that trans- isomers were degraded faster than the cis- isomers of similar compounds (4-methylcyclohexaneacetic acid, 4-methylcyclohexanecarboxylic acid and 3-methylcyclohexanecarboxylic acid). They suggest that the cis- isomer is more resistant because of the intramolecular hydrogen bonding. This assumption was based on adoption of the boat conformation. However the chair conformer of cyclohexane is favoured over the boat (the more stable twisted boat) conformer 10,000 to 1 at room temperature (Morrison and Boyd, 1992). Until the isomers have been separated in significant amounts for NMR analysis or studied by, for example, LC-NMR, it is difficult to assign the trans- and cis- isomers with any certainty.
Figure 3.11. Partial gas chromatograms of surrogate NA metabolites (as TMS esters) observed at day 30 following biodegradation using Devonport bacterial consortium (a) n-BCHBA, (b) iso-BCHBA, (c) sec-BCHBA and (d) tert-BCHBA. Chromatograms normalised to IS. (HP 5890 FID, Column, HP-1 30 m x 0.25 mm id x 0.25 µm film; oven programme; 40 – 300 °C at 10 °C min⁻¹, hold 10 min; injector 250 °C; detector 280 °C).
Figure 3.12. Partial gas chromatograms of n-BCHBA and metabolites (as TMS esters) recovered at each sampling point during biodegradation of the surrogate NA using the Devonport bacterial consortium. Chromatograms normalised to IS. (HP 5890 FID, Column, HP-1 30 m x 0.25 mm id x 0.25 μm film; oven programme; 40 – 300 °C at 10 °C min⁻¹, hold 10 min; injector 250 °C; detector 280 °C).
Figure 3.13. Partial gas chromatograms of *iso*-BCHBA and metabolites (as TMS esters) recovered at each sampling point during biodegradation of the surrogate NA using the Devonport bacterial consortium. Chromatograms normalised to IS. (HP 5890 FID, Column, HP-1 30 m x 0.25 mm id x 0.25 μm film; oven programme: 40 – 300 °C at 10 °C min⁻¹, hold 10 min; injector 250 °C; detector 280 °C).
Figure 3.14 shows the mean (± σ, n=3) production of the major metabolite for each of the surrogates. Mass spectra obtained for each of the major metabolites produced during biodegradation are shown in Figure 2.15. There were no significant differences in the mass spectra for the individual metabolite isomers, where resolved.

Figure 3.14. Percentage recovery of the major metabolites formed during biodegradation of (a) n-BCHBA, (b) iso-BCHBA, (c) sec-BCHBA and (d) tert-BCHBA (error bars represent ± σ, n=3; recovery is as a percentage of initial amount of substrate).
Figure 3.15. Electron impact mass spectra of major metabolites (as TMS esters) produced during the biodegradation of surrogate NAs using the Devonport bacterial consortium (a) \textit{n-BCHBA}, (b) \textit{iso-BCHBA}, (c) \textit{sec-BCHBA} and (d) \textit{tert-BCHBA}. (MS conditions: Ion source 250 °C, ionisation energy 70 eV, mass range 50 - 550 Daltons).
The degradation of each of the surrogates produced two major metabolite compounds consistent with the diastereo nature of the individual surrogate NAs, indicating that the two metabolites are likely diastereoisomers of the respective surrogate NAs. Production rates of the metabolites (n=3) correlated inversely with the degradation rate of the corresponding surrogate NA (Figures 3.10 to 3.14). Differences in retention times of the metabolites corresponded to the differences in retention times of the surrogates (i.e. tert-BCHBA with the lowest retention time and n-BCHBA with the highest retention time, likely a chromatographic effect of increased branching reducing the retention time).

Mass spectra of the metabolites (Figure 3.15) were all very similar with base peak ions of m/z 117, a fragmentation characteristic of TMS esters (derivative), and molecular ions of m/z 270, corresponding to a molecular weight of 198 Da for the free acids. The molecular weights of the metabolites were 28 Da less than that of the corresponding surrogate NAs, indicative of the loss of two CH₂ units. It is proposed that production of such metabolite follows β-oxidation routes reported previously (Quagrainé et al., 2005; Figure 3.16), and that the metabolites can be tentatively identified as 4-(4'-n-butylcyclohexyl)acetic acid, 4-(4'-iso-butylcyclohexyl)acetic acid, 4-(4'-sec-butylcyclohexyl)acetic acid and 4-(4'-tert-butylcyclohexyl)acetic acid (n-BCHAA, iso-BCHAA, sec-BCHAA and tert-BCHAA, respectively). The β-oxidation of fatty acids has been reviewed by Kunau et al., (1995). It is likely that aerobic bacteria degrade fatty acids via β-oxidation, including alkanes which are first converted to fatty acids by way of ω-oxidation. The authors suggest that E. coli can serve as a paradigm for fatty acid degradation in aerobic bacteria, and that in E. coli, eight genes encoding proteins required for the uptake (fadL), activation (fadL) and degradation (fadA, B, E, F, G and H) of medium-chain, long-chain, and unsaturated fatty acids have been identified and characterised (Table 3.5). The authors also report that fatty acid uptake has been proposed to occur by a facilitated process as well as by passive diffusion; this remains a subject of controversy.
The structures and identities of the metabolites could not be confirmed further herein as insufficient material was available for NMR spectroscopy. Similarly, comparison of the retention times and mass spectra of authentic samples to those of the hypothesised compounds was not possible during this study as \textit{n}-BCHAA, \textit{iso}-BCHAA, \textit{sec}-BCHAA and \textit{tert}-BCHAA are not commercially available and would have required synthesis, which time did not allow.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.16.png}
\caption{\textit{\(\beta\)}-oxidation of surrogate NAs (\(R = n\text{-}, iso\text{-}, sec\text{-} and tert\text{-}butyl\)). Adapted from Quagraine \textit{et al.}, (2005). Enzymes involved omitted for simplicity.}
\end{figure}
Table 3.5. Genes required for Fatty Acid degradation from *E. coli* (reproduced from Kunau et al., 1995).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>fadL</td>
<td>Fatty acid transporter (outer membrane)</td>
</tr>
<tr>
<td>fadD</td>
<td>Acyl-CoA synthetase</td>
</tr>
<tr>
<td>fadE</td>
<td>Electron transferring flavoprotein</td>
</tr>
<tr>
<td>fadF</td>
<td>Acyl-CoA dehydrogenase (long-chain)</td>
</tr>
<tr>
<td>fadG</td>
<td>Acyl-CoA dehydrogenase (short-chain)</td>
</tr>
<tr>
<td>fadB</td>
<td>Multifunctional β-oxidation protein, large subunit (enoyl-CoA hydratase,</td>
</tr>
<tr>
<td></td>
<td>3-L-hydroxyacyl-CoA dehydrogenase, 3-hydroxyacyl-CoA epimerase)</td>
</tr>
<tr>
<td>fadA</td>
<td>Multifunctional β-oxidation protein, small subunit (3-oxoacyl-CoA thiolase)</td>
</tr>
<tr>
<td>fadH</td>
<td>2,4-dienoyl-CoA reductase</td>
</tr>
<tr>
<td>fadR</td>
<td>Transcriptional regulator of <em>fad</em> genes</td>
</tr>
</tbody>
</table>

A minor metabolite was detected (Figure 3.11; RT 18.1, 17.5, and 17.9 min; present in trace amounts in *tert*-BCHBA) with a molecular ion of *m/z* 268 as a TMS ester, and therefore a molecular weight of 196 Da for the free acid (Figure 3.17). This would be consistent with the β-oxidation desaturase step in the further degradation of the BCHAA metabolites. Presumably degradation ceased at this stage due to hindrance from the ring (β carbon) preventing the following steps in the sequence.

![Minor metabolite](image)

**Figure 3.17** Minor metabolite formed from the β-oxidation desaturase step in the further degradation of the proposed major metabolite.

The degradation rate of the surrogate NAs suggests that the more branched the butyl side chain, the more resistant to degradation was the surrogate. It is well known that branched alkanes are more resistant to biodegradation than unbranched alkanes with the same formula (e.g. Garrett et al., 1999). However, the proposed β-oxidation pathway (Figure 3.16) does not involve the butyl side chain, suggesting that another mechanism controls the rate of degradation. An alternative explanation for control of the biodegradation rates may be the bioavailability of the surrogate NAs, with *tert*-BCHBA appearing to be the least bioavailable and *n*-BCHBA the most. A suggested explanation for the bioavailability of the
surrogates to the bacterial consortium is that the same process discussed in terms of toxicity is occurring (Section 2.2.2). In other words, \( n \)-BCHBA has the highest predicted log \( K_{ow} \) value and so is more efficient at partitioning through the cytoplasmic membrane of the bacteria (uptake route for small hydrophobic molecules; Brock et al., 1991) whereas \( tert \)-BCHBA has the lowest predicted log \( K_{ow} \) value and so is less efficient at crossing the membrane, and thus less bioavailable, resulting in a slower rate of degradation.

If hydrophobicity is an explanation of bioavailability this would help explain the results of previous studies where biodegradation of NA mixtures corresponded to a decrease in toxicity (Herman et al., 1994, Clemente et al., 2004, Clemente, 2004) as the most bioavailable components cause the highest level of toxicity, but they are also the least resistant to biodegradation.

Hermman et al., (1994) showed that pentylcyclohexane carboxylic acid (PCCA) was far more resistant to microbial attack than cyclohexane pentanoic acid (CHPA; 6 % mineralization in 24 days compared to 45 %). Findings in the current study are consistent with these results, where the carboxylic acid chain is attacked preferentially to an alkyl chain (note that uniquely, both moieties are present in each compound synthesised in this study). Hermman et al. (1994) considered CHPA to be a more representative NA surrogate as it is mineralised at a similar rate to some environmental NA mixtures. However, it could also be argued that PCCA is the more interesting surrogate as it is far more resistant to microbial attack and therefore of greater environmental concern.
3.2.5 4-(4'-iso-butylcyclohexyl)-4-methylbutanoic acid: Biodegradation study

In order to support the theory that the surrogate NAs were degraded via \( \beta \)-oxidation of the alkanoate chain, 4-(4'-iso-butylcyclohexyl)-3-methylbutanoic acid (iso-BCHMBA; Figure 3.18) was also synthesised and subjected to microbial attack using the Devonport consortium. The rationale for this study was that degradation via \( \beta \)-oxidation will not proceed if carbons \( \alpha \) or \( \beta \) with respect to the carbonyl group contain methyl branches, as the enzymatic transformation cannot occur (Alexander, 1999). Similarly a methyl branch on the \( \gamma \) carbon would prevent \( \beta \)-oxidation and would hinder the formation of the resulting carboxylic acid. The presence of a methyl group positioned on the \( \alpha \) carbon with respect to the ring is also consistent with the recent assignment of a partial structure of the so-called ARN acid. ARN acids are a group of 4-protic carboxylic acids containing 4 to 8 unsaturated sites (rings) in the hydrocarbon skeleton which have been identified as the dominating constituents of the calcium naphthenate deposits which cause huge problems during production and refining of oil (Baugh et al., 2005).

A further rationale for the synthesis and study of iso-BCHMBA, which has a methyl branch on the \( \gamma \) carbon, was that it was a synthetically more viable proposition than the corresponding isomers with the branch point on the \( \alpha \) or \( \beta \) carbons with respect to the carbonyl group.

It was hypothesised that branching at any point of the butanoic acid chain of the surrogate NAs would initially stop degradation by \( \beta \)-oxidation, and so, by implication, confirm the proposed \( \beta \)-oxidation metabolic pathway for the degradation of \( n \)-BCHAA, iso-BCHAA, sec-BCHAA and tert-BCHAA. Iso-BCHMBA was chosen, as opposed to \( n \)-, sec- or tert-BCHMBA, as previously iso-BCHBA showed a significant rate of degradation over 30 days that would allow comparison over a 40 day period (Figure 3.10b, page 146).

Figure 3.19 shows a histogram of the results of the biodegradation of iso-BCHBA and iso-BCHMBA. The biodegradation of iso-BCHBA (Figure 3.10b) was repeated in this experiment (Figure 3.19) and examined parallel to that of iso-BCHMBA so that their rates
of degradation could be accurately compared. Procedural blanks and abiotic controls were also examined. All samples were examined in triplicate, allowing standard deviations to be calculated. It can be seen from the histogram that by day 20 there was a significant difference in degradation rates between iso-BCHMBa and iso-BCHBA \((P = 0)\), and also at day 42 \((P = 0)\). In fact there was no biodegradation of iso-BCHMBa, even after 42d. The production of 4-(4′-iso-butylecylhexyl)acetic acid as the major metabolite was seen at day 20 and 42 in the iso-BCHBA samples \((cf\ Figure\ 3.14b,\ page\ 152)\), whereas no metabolites were seen in the experiment conducted with iso-BCHMBa, further confirming that no biodegradation had occurred.

These results support the suggestion that the metabolic pathway for the degradation of \(n\)-BCHAA, iso-BCHAA, sec-BCHAA and tert-BCHAA was \(\beta\)-oxidation and that the methyl branch in iso-BCHMBa had hindered this pathway.

![Figure 3.18](image)

**Figure 3.18.** 4-(4′-iso-butylecylhexyl)-4-methyl-butanoic acid (iso-BCHMBa). \(\alpha\), \(\beta\) and \(\gamma\) carbons labelled with respect to the carbonyl group, and \(\alpha^1\) with respect to the ring.

![Figure 3.19](image)

**Figure 3.19.** Biodegradation of iso-BCHMBa compared to iso-BCHBA by the Devonport consortium over 42 days. (** indicates a significant difference greater than \(P = 0.001\).)
3.3 Conclusions

The aim of the present study was to investigate the toxicity of the synthesised isomeric monocyclic surrogate naphthenic acids and to compare the results in order to examine the effect of branching of the butyl substituents. A further aim was to investigate the resistance to biodegradation of the surrogates which are uniquely different to those used in previous studies as their structure possesses a cyclic moiety with both alkyl and alkanoic substituents.

The initial toxicity study showed the monocyclic synthetic NAs to be less toxic to developing oyster embryos than a bicyclic surrogate which in turn was less toxic than a polycyclic surrogate. The detailed toxicity study of the monocyclic isomeric series of surrogate NAs showed n-BCHBA to be the most toxic (EC50 0.11 mg L⁻¹) and tert-BCHBA to be the least toxic (EC50 0.49 mg L⁻¹; iso-BCHBA EC50 0.21 mg L⁻¹, sec-BCHBA EC50 0.33 mg L⁻¹). The results show a QSAR in which the branching of the alkyl (butyl) substituent influences the toxic effect to the developing embryos, with increased branching correlating with a decreased toxic effect. The QSAR has been attributed to the aqueous solubility of the surrogates (estimated using Kowwin, EPI suite) where increasing water solubility accompanies a decreasing toxic effect.

The results of the mixed culture biodegradation study showed a large variation in degradation rates between the synthetic monocyclic surrogates. n-BCHBA was the most readily degraded with 97% degradation by day 9, and tert-BCHBA the most resistant with only 2% degradation by day 30. iso-BCHBA and sec-BCHBA were 77% and 53% degraded after 30 days, respectively. The degradation of the surrogates coincided with the production of a metabolite consistent with a β-oxidation attack to the alkanoic chain. The degradation via β-oxidation was supported by the resistance to biodegradation of iso-BCHMBA, where the methyl branching of the alkanoic chain hindered the biodegradation pathway.
The results of the toxicity and biodegradation studies indicate a link between the toxicity and biodegradation of the surrogates and their structure. It is proposed that the degree of toxicity and resistance to biodegradation are linked to the surrogate structures in a QSAR affected by their hydrophobicity, and thus determining their bioavailability. This link is consistent with previous studies where initial biodegradation of natural NAs has decreased toxicity (Herman et al., 1994). However, of important note is that although the present study shows the most toxic surrogate (n-BCHBA) to be readily degraded, the most recalcitrant surrogate (tert-BCHBA) still exhibited a toxic effect of the same order of magnitude. Thus bioresistant NAs would perhaps also be expected to be toxic.

The current study indicates that ‘real’ NAs are likely to comprise cyclic compounds with either acetic acid groups, or branched alkanoate chains (e.g. Figure 3.20 a and b, respectively) and additional branched alkyl chains substituents. Thus, bioremediation of recalcitrant NAs is reliant on finding bacteria that will degrade these types of compounds.

![Figure 3.20](image)

**Figure 3.20.** Hypothesised ‘real’ NA structures. Examples are monocyclic for simplicity, although maybe bi-, or polycyclic. Branched alkyl substituent represented by tert-butyl.
Chapter 4 Development of high performance liquid chromatography (HPLC) separation of naphthenic acids with detection by electrospray ionisation mass spectrometry (ESI-MS)

4.1 Introduction

Electrospray ionisation (ESI) mass spectrometry is a technique which is widely used for pharmaceutical and biomedical applications, replacing techniques such as fast atom bombardment (FAB), thermospray (TSP), and particle beam (PB) mass spectrometry for the analysis of thermally labile, polar and ionic compounds (Roussis and Fedora, 2002, and references therein). In the coupling of LC to MS, the ESI source serves the purpose both of an LC/MS interface and a means of generating ions (Dass, 1999). The removal of all but the gas phase ions whilst remaining at atmospheric pressure makes ESI one of the most important ionisation techniques for the on-line coupling of liquid phase separation techniques such as LC with MS (Smyth, 2005).

4.1.1 Electrospray ionisation mass spectrometry (ESI-MS)

The earliest reports of coupling electrospray ionisation and mass spectrometry were independently presented by Yamashita and Fenn, (1984), and almost concurrently by Aleksandrov et al. (1984). The importance of the technique was recognised by the scientific community in 2002 when Fenn received a share of the Nobel Prize in Chemistry for his pioneering work in electrospray ionisation, for which he gave an acceptance lecture titled ‘Electrospray Wings for Molecular Elephants’ (http://nobelprize.org/chemistry/laureates/2002/index.html). The technique is now widely used for analysis ranging from high molecular-mass compounds such as proteins, nucleotides and synthetic polymers to low molecular-mass molecules such as drugs and their metabolites (Smyth, 2003, 2005, Rosenberg, 2003).

A simplified diagram of the Finnigan Matt LCQ™ (used in the current study) is shown in Figure 4.1. The process of ESI-MS has been excellently described and reviewed by Cech and Enke (2001), amongst others, and indeed the process of ESI in conjunction with
quadrapole ion-trap MS in context of the Finnigan Matt LCQ™ has been described by Bier and Schwartz (Cole, 1997), and more recently by M'Cormack (2003). It is not the intention to reproduce those excellent reviews here, but briefly to describe the principles of ESI-MS as used in the current study. Briefly, the analyte is introduced through an electrospray capillary to which a high potential is applied (3-5 kV). A gradient caused between the high voltage of the electrospray capillary and the low voltage applied to the heated capillary sampling orifice of the mass spectrometer creates a potential which causes ions of the same polarity to concentrate at the electrospray capillary tip. The gradient causes the ions to be drawn out of the capillary tip forming a 'Taylor cone', where the concentration of ions increases at the surface to a point where the cumblic forces over come the surface tension causing charged droplets to be emitted. The charged droplets are then drawn towards the sampling orifice by both the potential gradient and the vacuum created by the sampling orifice of the high vacuum of the mass spectrometer. Solvent evaporation and shrinkage, aided by a coaxial flow of nitrogen, causes the increased concentration of ions of similar polarity at the liquid surface, to a point where Cumblic forces again over come the surface tension resulting in droplet disintegration. The droplets disintegrate to a point where the they are very small highly charged droplets that can produce gas phase ions (Cech and Enke, 2001, M'Cormack, 2003). The resulting ions are mostly pseudomolecular ions with very little, or no, fragmentation caused by the soft ionisation process.

ESI has been interfaced with many mass analysers including single (Q) and triple (QqQ) quadrapole, quadrapole ion traps (QIT), time of flight (TOF), sector and Fourier transform ion cyclotron resonance (FT-IRC) instruments (M'Cormack, 2003, and references therein). The present study utilised a Finnigan Matt LCQ™ QIT instrument as detailed by Bier and Schwartz (1997). The ions produced by the ESI source are focused through a skimmer and two octapole lenses to the ion trap which consists of three electrodes, two hyperbolic end caps and a central hyperbolic ring electrode. The instrument uses radio frequency (RF) and
direct current (DC) voltages to transport, separate and scan the ions. Once the ions have been injected to the ion trap they can be isolated and stored for a given amount of time before being ejected according to increasing m/z, to the detector. The isolation of ions allows operations such as collision-induced dissociation (CID) MS² to be carried out in the same analyser, where by an ion is further fragmented by collision with helium (so-called multistage MS or MS²).

![Diagram of Finnigan Matt LCQ™ electrospray ionisation source with droplet and gas phase ion production](image)

**Figure 4.1.** Simplified diagram of the Finnigan Matt LCQ™ electrospray ionisation source with droplet and gas phase ion production (reproduced with permission from Mc Cormack, 2003).

### 4.1.2 ESI-MS in naphthenic acid research

Although the use of ESI-MS and LC/MS is now extensive in the recent literature, relatively few studies have involved naphthenic acids, and of the few studies which have, little or no structural characterisation of unknown compounds has been reported. Rudzinski *et al.* (2002) performed ESI-MS/MS experiments on a range of authentic acids and on two commercial naphthenic acid samples. The ESI-MS/MS of authentic acids used
a negative ion mode at concentrations of 10 mg mL\(^{-1}\) (10,000 ppm), yielding fragmentation patterns that verified the presence of functional groups such as carboxyl. The analysis of commercial naphthenic acid samples allowed identification of Z series and carbon number and verification of carboxyl and sulfanoic moieties. Headley \textit{et al.}, (2002) used an SPE method to concentrate naphthenic acids from aqueous samples prior to negative ion ESI-MS analysis of mixtures to a detection limit of 0.01 mg L\(^{-1}\) (10 ppb). No fragmentation or characterisation data were presented. Lo \textit{et al.}, (2003) calibrated a negative ion ESI-MS method using six carboxylic acids. They used the calibrated method to analyse authentic NAs showing 'congener' distributions (Z and carbon numbers) which they concluded were similar to those observed by GC-MS. Once again, no fragmentation or characterisation data were presented. Hsu \textit{et al.}, (2000) showed negative ion atmospheric pressure ionisation (APCI) to be an order of magnitude more sensitive than negative ion ESI, and used APCI to show Z and carbon number distributions. No data were presented for concentrations or limits of detection. Conversely, Roussis and Fedora (2002) compared the detection limits and linear dynamic ranges of ESI and APCI over concentrations of 1 – 100 ppm. They found the detection limit of ESI to be approximately two orders of magnitude higher than that of APCI, and both to have approximately the same relative linear dynamic range. Perhaps the greatest information about the structures of NAs was achieved using ESI-high field asymmetric waveform ion mobility spectrometry (FAIMS) combined with quadrupole, time-of-flight, and tandem mass spectrometry (Gabryelski and Froese, 2003). The authors presented a method to analyse NA mixtures without extensive sample preparation, which allowed them tentatively to assign two structures to NAs from an oil sand extract. They commented that characterisation of new structures remains the “most challenging part of NA investigation” and that a lack of representative authentic compounds is an issue \textit{(cf Chapter 2, herein)}.

ESI in conjunction with Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) has been used to provide accurate mass measurements which allow
determination of elemental composition of naphthenic acids. The elemental composition is used to predict the structure based on common understanding of possible configurations. However, this approach does not provide conclusive information on molecular structures (Qian and Robbins, 2001; Hughey et al., 2002; Barrow et al., 2003; Wu et al., 2004; Barrow et al., 2004).

4.1.3 Detection orientated derivatisation

The sensitivity of mass spectrometry is dependent on the formation of ions. Although carboxylic acids form ions readily in solution, these ions have tendencies not be transferred and detected by the mass spectrometer because of their affinity to form adducts with other ions and contaminants, thus suppressing their detection. This has led to derivatisation of carboxylic acids to enhance their detection for chromatographic analysis (Knapp, 1979; Lunn and Hellwig, 1998; Cartwright et al., 2005). Previous derivatives have involved the inclusion of fluorescent tags (Toyo’oka, 2002) or of functional groups suitable for electrochemical detection methods (Mortia and Konishi, 2003; Cartwright et al., 2005). Although the electrochemical derivatives enhance detection, their quite often bulky nature is not well suited to characterisation of unknown structures, such as those of NAs.

Few studies have focused on developing HPLC methods primarily for the separation and detection of NAs. Clemente et al. (2003a) and Yen et al. (2004) reported the use of 2-nitrophenylhydrazine derivatives which they injected onto a reverse phase C18 column. The NAs eluted as unresolved complex mixtures which were quantified to monitor biodegradation studies.

4.1.4 Current aims

The aim of the present study was to develop a more suitable derivative that would increase detection response of NAs by ESI-MS, and would enable MS" characterisation. Conversion of NAs to amides was chosen as amides generally have good detection response in positive ESI-MS, whilst at the same time, do not significantly change the molecular structure of the
NA. The derivatisation of NAs and then HPLC/ESI-MS separation and MS\textsuperscript{n} was initially developed using novel synthesised acids (Chapter 2), prior to analysis of complex mixtures of unknown, natural NAs.

4.2 Results and discussion

4.2.1 Amide derivatisation

Preliminary ESI-MS characterisation studies had shown that NAs gave very poor detection response in the present system. Therefore, conversion of NAs to amides was chosen on the basis of previous work at the University of Plymouth (McConnack, unpublished data) which showed amides gave good detection response by ESI-MS. NAs were converted to naphthenic amides (NAds), in quantitative yields, via acid chlorides by treatment with oxalyl chloride to form the corresponding NA chlorides, which were then treated with ammonia to form the NAds. Oxalyl chloride was chosen as it gives high yields and reacts under milder conditions than alternatives such as thionyl chloride and phosphorous trichloride (McMurry, 1988). A detailed experimental procedure is given in Chapter 5 (Section 5.8). Infrared spectroscopy was used to confirm the conversion of a carboxylic acids to amides. An example is shown in Figure 4.2 for cyclohexylbutanamide (CHBAd). The figure shows the spectrum of CHBAd overlaid on the spectrum of cyclohexylbutanoic acid (CHBA), highlighting the change in absorbances characteristic of the conversion of carboxylic acid group to an amide. Of particular significance is the appearance of strong absorbances at 3370 and 3189 cm\textsuperscript{-1} due to the \(-\text{NH}_2\) stretching and the shift of carbonyl stretching from 1706 to 1645 and 1632 cm\textsuperscript{-1} (Williams and Fleming, 1973).
To evaluate the derivatisation of NAs to amides, infusion ESI analysis was performed on cyclohexylbutanoic acid and cyclohexylbutanamide as a direct comparison using the same solvent conditions with appropriate modifiers to aid (de)protonation (Chapter 5). The negative ion full scan mass spectra of cyclohexylbutanoic acid (Figure 4.3) shows a base ion of \( m/z \ 169.3 \) which corresponds to the \([M-H]^\text{-}\) ion, while the positive ion full scan for cyclohexylbutanamide (Figure 4.4) shows a base ion of \( m/z \ 170.3 \) corresponding to the \([M+H]^+\) ion. It can be seen that the detection response improved by two orders of magnitude (2.83E4 to 3.83E6) following conversion to the amide. Under the conditions applied the \([M-H]^\text{-}\) ion of the acid did not fragment to produce MS\^{2} \ spectra, while the \([M+H]^+\) ion of the amide produced reproducible ions for several stages of MS (MS\^{n}; Section 4.2.2.1).
Figure 4.3. Infusion ESI-MS mass spectrum of cyclohexylbutanoic acid (10 ppm). Negative ion mode, full scan m/z 100–500.

Figure 4.4. Infusion ESI-MS mass spectrum of cyclohexylbutanamide (10 ppm). Positive ion mode full scan m/z 100–500.
4.2.2 Infusion electrospray ionisation multistage mass spectrometry (ESI-MS") analysis of known amides

The amides of the previously synthesised surrogate NAs (Chapter 2) were examined by infusion ESI-MS" to better understand the MS" fragmentation pathways that might be utilised for the molecular characterisation of unknown NAds. Establishment of the proposed pathways was aided by the results of preliminary studies (McCormack, unpublished data).

4.2.2.1 Infusion electrospray ionisation multistage mass spectrometry (ESI-MS"):
Analysis of cyclohexylbutanamide

The MS full scan mass spectrum of cyclohexylbutanamide (Figure 4.5A) shows a base ion (m/z 170.3) which corresponds to the protonated amide ([M+H]+). The ZoomScan spectrum (Figure 4.5B) shows a resolution of m/z 1 between 12C and 13C isotopes which indicates that the ion is singularly charged, as expected for the protonation of the single nitrogen. MS2 CID fragmentation (Figure 4.5C) of the precursor ion (m/z 170.3) gave rise to two prominent product ions at m/z 135.1 (base ion) and m/z 88.1 (~90 %), and less abundant product ions at m/z 152.2 (~20 %) and m/z 107 (~10 %). The product ions, m/z 135.1 and 88.1, were also observed at a very low intensity (~5 %) in MS1 (Figure 4.5A) resulting from in-source fragmentation of [M+H]+. From these data the following CID fragmentation pathway is proposed (Figure 4.6) whereby the amide is first dehydrated to form a ketenimine ion from the loss of water (II; m/z 152.2; pathway a) which then loses ammonia (NH3) to form a carbocation (IV; m/z 135.1; pathway e). The carbocation IV (m/z 135.1) is also formed via an oxonium ion (III; m/z 153.2; pathway f) resulting from elimination of NH3 from the protonated amide (pathway b). The ion at m/z 88.1 (~90 %) is thought to be a fragment ion (V) resulting from cleavage of the butanamide substituent from the precursor ion (I; pathway d). The MS3 spectrum (Figure 4.5D) of MS2 ion m/z 135.1 (Figure 4.5C) shows further fragmentation of the alkyl moiety of IV. The base peak (VIII; m/z 107.18; pathway h) results from the loss of ethene, while the less intense product ions (<7 %) are due to cleavage and rearrangement of the cyclic moiety.
The MS² ion m/z 135.1 (loss of 35) is characteristic of an amide with loss of H₂O and NH₃, and the ion at m/z 88.1 is indicative of the cleavage of butanamide from the ring moiety.

**Figure 4.5.** Infusion ESI-MS mass spectra of 4-cyclohexylbutanamide in positive ion mode (10 ppm; A) MS¹, (B) ZoomScan of precursor ion m/z 170.2 (C) MS² spectrum of MS¹ ion m/z 170.2 (27 % AA), (D) MS³ spectrum of MS² ion m/z 135.1 (26 % AA) and (E) MS⁴ spectrum of MS³ ion m/z 107.1 (23 % AA).
Figure 4.6. Proposed CID fragmentation pathway for 4-cyclohexylbutanamide.
4.2.2.2 Infusion electrospray ionisation multistage mass spectrometry (ESI-MS"): Analysis of 2-(4'-methylcyclohexyl)acetamide

The MS full scan mass spectrum of 2-(4'-methylcyclohexyl)acetamide (Figure 4.7A) shows a base ion (m/z 156.1) which corresponds to the protonated amide ([M+H]+). As with the previous compound (cyclohexylbutanamide; I; section 4.2.2.1), resolution between the 12C and 13C isotopes in the ZoomScan spectrum (Figure 4.7B) is indicative of a singularly charged ion. MS² CID fragmentation (Figure 4.7C) of the precursor ion (m/z 156.1) produces a base ion at m/z 96.9 and further prominent ions at m/z 121.0 (~80 %), m/z 139.0 (~50 %) and m/z 110.9 (~45 %). The proposed fragmentation pathway (Figure 4.8), following the analysis of the MS² spectra, can be deduced if the amide first loses ammonia to form an oxonium ion (XII; m/z 139.0; pathway b) which then loses water to form an MS³ carbocation (XIII; m/z 121.0; pathway f). MS⁴ CID fragmentation of XIII results in a base ion (m/z 93.1) proposed to result from the cleavage at the ring and the loss of ethene (XVI; pathway j). The pathway for XI to XVI (b, f and j) is the same as that for I to IX in the CID fragmentation of 4-cyclohexylbutanamide (I; Figure 4.6). The base ion in the MS² CID fragmentation of the precursor ion is proposed to be due to cleavage of acetamide at the ring moiety (XV; m/z 96.9; pathway n) while the ion at m/z 121.20 is formed via pathway c as seen in the fragmentation of 4-cyclohexylbutanamide. The MS² CID fragmentation base ion (XV; pathway n) is formed via a different pathway to the base ion in MS² CID fragmentation of 4-cyclohexylbutanamide (I; Figure 4.6), and is unique to the acetamide moiety of 2-(4'-methylcyclohexyl)acetamide (XI). Therefore ions resulting from pathway n (i.e. loss of m/z 59) in MS² spectra of unknown amides are likely to be indicative of ethanoate moieties in the original NAs.
Figure 4.7. Infusion ESI-MS mass spectra of 2-(4'-methylocyclohexyl)acetamide in positive ion mode (A) MS\(^1\), (B) ZoomScan of \(m/z\) 156.1 (C) MS\(^2\) spectrum of MS\(^1\) ion \(m/z\) 156.1 (30 % AA), (D) MS\(^1\) spectrum of MS\(^2\) ion \(m/z\) 139.0 (27 % AA) and (E) MS\(^3\) spectrum of MS\(^1\) ion \(m/z\) 121.0 (25 % AA).
Figure 4.8. Proposed CID fragmentation pathway for 2-(4'-methylcyclohexyl)acetamide.
4.2.2.3 Infusion electrospray ionisation multistage mass spectrometry (ESI-MS$^n$): Analysis of 4-(4-butylecyclohexyl)butanamide isomers

The infusion ESI-MS mass spectra of iso-BCHBAAd, n-BCHBAAd, sec-BCHBAAd and tert-BCHBAAd are shown in Figure 4.9, 4.10, 4.11 and 4.12 respectively. The MS$^n$ spectra (C-F in each respective figure) for each isomer exhibit all the same fragmentation ions, with no ions unique to one individual isomer. This suggests that the butyl chain isomerism is not directly involved in any of the observed fragmentation pathways. However, Figure 4.14 demonstrates that while all the ions present have the same mass/charge ratio for each isomer, the intensities differ. This indicates that although the isomeric butyl chains are not directly involved in any observed fragmentation pathways, they do affect the stability of the fragment ions. In order to comment on trends in stabilities of the fragment ions for each isomer, the CID fragmentation pathways were first analysed and structures proposed (Figure 4.13).

The MS$^2$ ion $m/z$ 88.1 is thought to be the same ion as that seen in the MS$^2$ spectrum of cyclohexylbutanamide (Section 4.2.2.1; V; pathway d) resulting from the cleavage of the butanamide moiety. The ion is in high abundance (<60%) for each isomer. The MS$^2$ ion observed at $m/z$ 208.2 is formed via pathway b, and is thought to correspond to a ketenimine ion (XVIII), which in MS$^3$ CID fragmentation loses ammonia forming a carbocation (XIX; pathway e). The carbocation XIX is also observed in MS$^2$ (pathway e). The MS$^3$ carbocation XIX is then thought to undergo a charge remote fragmentation whereby the butyl moiety is lost (pathway t) resulting in XX ($m/z$ 135.1). XX is formed by the same pathway (t) during MS$^3$ CID fragmentation of the MS$^2$ ion $m/z$ 191.1 (XIX). MS$^4$ fragmentation of XX results in the fragment ions $m/z$ 107.0, 93.1 and 79.1 (VII, VIII, and IX; pathways h, i and j, respectively). These ions are confirmation that XX is indeed formed by a charge remote fragmentation from XIX, (i.e. cleavage of the alkyl chain (butyl) and not the butyl carbocation) as VII, VIII and IX would not be possible with all isomers if the XX was formed by cleavage of the butyl carbocation moiety from XIX. The carbocation, XXI, formed by pathway p in the MS$^2$ CID fragmentation of MS$^1$ ion $m/z$
226.3 (and also pathways r and u in latter MS^n stages) has not yet been identified, but due to subsequent MS^3 CIS fragmentation (pathway v) is thought to involve a charge remote fragmentation cleavage of the butyl moiety as in the formation of XIX.

A stable ion resulting from pathway d (i.e fragment ion m/z 88.1) in the MS^2 spectrum of an unknown amide is likely indicative that the alkenoate moiety in the original NA is butanoate.

The major difference in relative intensities of the CID MS^n ions for the 4 isomers involves the ions m/z 191.1 (XIX), m/z 135.1 (XX) and m/z 121.1 (XXI). The differences in intensities are thought to be due to the different stabilising effects on the fragment ions, although no pattern could be defined for different butyl chain isomers. Therefore, with the current knowledge of ESI fragmentation, differentiation between the isomeric forms (side chain branching) of alkyl substituents of NAds is not possible.
Figure 4.9. Infusion ESI-MS mass spectra of iso-BCHBAd in positive ion mode (A) MS$^1$, (B) ZoomScan of ion m/z 226.3 (C) MS$^2$ spectrum of MS$^1$ ion m/z 226.5 (29 % AA), (D) MS$^1$ spectrum of MS$^2$ ion m/z 208.2 (27 % AA), (E) MS$^4$ spectrum of MS$^1$ ion m/z 191.1 (23 % AA) and (F) MS$^4$ spectrum of MS$^3$ ion m/z 135.1 (24 % AA).
Figure 4.10. Infusion ESI-MS mass spectra of n-BCHBAAd in positive ion mode (A) MS\(^1\), (B) ZoomScan of ion \(m/z\) 226.2 (C) MS\(^2\) spectrum of MS\(^1\) ion \(m/z\) 226.5 (28 % AA), (D) MS\(^3\) spectrum of MS\(^2\) ion \(m/z\) 208.2 (27 % AA), (E) MS\(^4\) spectrum of MS\(^3\) ion \(m/z\) 191.1 (24 % AA), (F) MS\(^4\) spectrum of MS\(^3\) ion \(m/z\) 135.1 (24 % AA) and (G) MS\(^4\) spectrum of MS\(^3\) ion \(m/z\) 121.1 (22 % AA).
Figure 4.11. Infusion ESI-MS mass spectra of sec-BCHBA in positive ion mode (A) MS\(^1\), (B) ZoomScan of ion m/z 226.2 (C) MS\(^2\) spectrum of MS\(^1\) ion m/z 226.5 (28 % AA), (D) MS\(^3\) spectrum of MS\(^2\) ion m/z 208.2 (27 % AA), (E) MS\(^4\) spectrum of MS\(^3\) ion m/z 191.1 (24 % AA), (F) MS\(^4\) spectrum of MS\(^3\) ion m/z 135.1 (24 % AA) and (F) MS\(^4\) spectrum of MS\(^3\) ion m/z 121.1 (22 % AA).
Figure 4.12. Infusion ESI-MS mass spectra of tert-BCHBAD in positive ion mode (A) MS\(^1\), (B) ZoomScan of ion \(m/z\) 226.3 (C) MS\(^2\) spectrum of MS\(^1\) ion \(m/z\) 226.6 (30 % AA), (D) MS\(^3\) spectrum of MS\(^2\) ion \(m/z\) 208.2 (27 % AA), (E) MS\(^4\) spectrum of MS\(^3\) ion \(m/z\) 191.1 (22 % AA), (F) MS\(^4\) spectrum of MS\(^3\) ion \(m/z\) 135.1 (24.5 % AA) and (G) MS\(^4\) spectrum of MS\(^3\) ion \(m/z\) 121.1 (22 % AA).
Figure 4.13. Proposed CID fragmentation pathways for BCHBAd isomers.
Figure 4.14. MS² spectra of MS¹ ions m/z 226 for (A) iso-BCHBAd (AA = 29%), (B) n-BCHBAd (AA = 28%), sec-BCHBAd (28%) and tert-BCHBAd (30%).
4.2.2.4 Infusion electrospray ionisation multistage mass spectrometry (ESI-MS²): Analysis of adamantane carboxamide

Adamantane carboxamide was used as an internal standard in analysis of NA mixtures, therefore the ZoomScan spectrum (Figure 4.15) contains ions originating from other NAs and not just the $^{12}\text{C}/^{13}\text{C}$ isotope ions of the compound. However, importantly it can be seen that there is base-line resolution with a peak width of $m/z$ 1 between the $^{12}\text{C}/^{13}\text{C}$ isomers, indicative of singularly charged ions. MS² CID fragmentation of the MS¹ ion $m/z$ 180.2 (Figure 4.15C) resulted in a single fragment ion at $m/z$ 135.1 (XXII). The carbocation XXII is thought to be formed by cleavage of formamide from the adamantane ring (pathway w) and not via a ketenimine ion as with other surrogate NAds. The direct cleavage of the carboxylic amide moiety arises because the quaternary $\alpha$ carbon does not permit the formation of the ketenimine ion. MS³ CID fragments of the MS² adamantane carbocation (XXII) yielded ions at $m/z$ 107.0 (XXIV; pathway y), $m/z$ 93.1 (XXV; pathway z) and $m/z$ 79.0 (IX; pathway x; Figure 4.16).
Figure 4.15. Infusion ESI-MS mass spectra of adamantane carboxamide as part of a mixture in positive ion mode (A) MS$^1$, (B) ZoomScan of ion m/z 180.2 (C) MS$^2$ spectrum of MS$^1$ ion m/z 180.2 (32% AA) and (D) MS$^3$ spectrum of MS$^2$ ion m/z 135.1 (25% AA).
Figure 4.16. CID fragmentation pathway for adamantane carboxamide.
4.2.2.5 Infusion electrospray ionisation multistage mass spectrometry (ESI-MS²): Analysis of decahydro-2-naphthanamide (DHNAd)

MS² CID fragmentation of the MS¹ ion m/z 182.3 (Figure 4.18C) results in a base ion at m/z 137.0 and less abundant ions at m/z 165.2, 164.2, 147.1 and 81.1. It is thought that the protonated amide (XXVI) forms a carbocation (XXVIII; Figure 4.18) via pathways a and e (via a ketenimine ion) and b and f (via an oxonium ion). The carbocation (XXVIII) then undergoes further CID fragmentation to yield carbocations m/z 119.1 (XXX; pathway β) and m/z 105.1 (XXXI; pathway γ). The MS² CID fragmentation base ion (m/z 137.0) is thought to result from the cleavage of formamide from the bicyclic moiety, resulting in a stable bicyclic carbocation (XXXIII; pathway w). Therefore, a stable ion resulting from pathway w (i.e. loss of m/z 45) in an unknown amide MS² spectrum is likely indicative that the amide is attached directly to a cyclic moiety.
Figure 4.17. Infusion ESI-MS mass spectra of DHNAld in positive ion mode (A) MS\textsuperscript{1}, (B) ZoomScan of ion m/z 182.30 (C) MS\textsuperscript{2} spectrum of MS\textsuperscript{1} ion m/z 182.3 (28.5 % AA), (D) MS\textsuperscript{3} spectrum of MS\textsuperscript{2} ion m/z 164.2 (24 % AA), (E) MS\textsuperscript{4} spectrum of MS\textsuperscript{3} ion m/z 147.1 (22 % AA) and (F) MS\textsuperscript{3} spectrum of MS\textsuperscript{2} ion m/z 137.0 (24 % AA).
Figure 4.18. CID fragmentation pathway for decahydro-2-naphthanamide (DHNAd).
4.2.2.6 Infusion electrospray ionisation multistage mass spectrometry (ESI-MS^n):

Analysis of cholamamidé

MS^2 CID fragmentation (Figure 4.19C) of MS^1 ion m/z 360.5 gave rise to a base ion at m/z 343.3, less abundant ions at m/z 342.3 and 325.3, and a series of ions between m/z 150-264.

The proposed MS^2 CID fragmentation pathway (Figure 4.20) is that the amide forms a carbocation at m/z 325.3 (XXXVI; pathway c) via either a ketenimine ion (m/z 344.3; XXXXII; pathways a and e) or an oxonium ion (m/z 343.3; XXXVII; pathways b and f).

MS^3 fragmentation shows losses of 14 Da, indicative of successive losses of methylene groups.
Figure 4.19. Infusion ESI-MS mass spectra of cholanamide in positive ion mode (A) $MS^1$, (B) ZoomScan of ion $m/z$ 360.3, (C) $MS^2$ spectrum of $MS^1$ ion 360.5 (37 % AA) and (D) $MS^3$ spectrum of $MS^2$ ion $m/z$ 325.3 (29 % AA).
Figure 4.20. CID fragmentation pathway for cholamidide.
4.2.2.7 Summary of NAd ESI-MS\textsuperscript{n} CID fragmentation patterns

ESI-MS\textsuperscript{n} CID fragmentations of NAd are characterised by initial losses of \textit{m/z} 17, 18 and 35. The protonated amide (\([\text{M+H}^+]\)) initially eliminates either H\textsubscript{2}O (\([\text{M+H}^+ - 18]\)) to form a ketenimine ion (pathway a), or NH\textsubscript{3} (\([\text{M+H}^+ - 17]\)) to form an oxonium ion (pathway b) during MS\textsuperscript{2} CID fragmentation. The ketenimine and oxonium ions are of low to medium relative abundance (<50 %), and the ratio between them varies with no identifiable structural correlation. The ketenimine ion then eliminates NH\textsubscript{3} to form a carbocation (\([\text{M+H}^+ - 35]\); pathway e) of high relative abundance (50 - 100 %) during MS\textsuperscript{3} CID fragmentation. The same carbocation is also produced by the elimination of H\textsubscript{2}O from the oxonium ion (pathway f) in MS\textsuperscript{2}. The \([\text{M+H}^+ - 35]\) carbocation was also observed during MS\textsuperscript{2} CID fragmentation (pathway e). Adamantane carboxamide proved to be the exception where the presence of a quaternary \(\alpha\) carbon did not allow the formation of either an oxonium or a ketenimine ion, or indeed the subsequent carbocation (pathways a + e and b + f). Instead, an MS\textsuperscript{2} base peak ion at \([\text{M+H}^+ - 45]\) was observed and thought to be a carbocation resulting from the cleavage of the formamide moiety from the ring structure (pathway w). This was also observed in the MS\textsuperscript{2} spectrum of decahydro-2-naphthoic acid which also exhibited a \([\text{M+H}^+ - 45]\) base ion. Similarly, an MS\textsuperscript{2} base ion of \([\text{M+H}^+ - 59]\) was observed for 2-(4'-methylcyclohexyl)acetamide resulting from the cleavage of acetamide from the cyclic moiety (pathway n). The MS\textsuperscript{2} fragment ion V (\textit{m/z} 88.1; pathway d) was observed in moderate intensity (>50 %) for only the five NAd with a butanamide moiety, thus the occurrence of V is indicative of butanamide substituted NAd.

In the identification of unknown NAd from a complex mixture, no one compound will probably ever be isolated sufficiently pure to allow infusion MS\textsuperscript{n} CID fragmentation. However, following HPLC separation, the operating software allows data dependent MS\textsuperscript{2} CID fragmentation yielding MS\textsuperscript{2} spectra of individual NAd. Therefore structural identification must be achieved through MS\textsuperscript{2} CID fragmentation alone. The common losses observed in the MS\textsuperscript{2} CID fragmentation of unique NAd standards are shown in Table 4.1. 193
An MS$^2$ spectrum of an unknown NAd should initially allow confirmation that the compound is an amide through identification of the characteristic pathways listed in Table 4.1. Determination of molecular mass will allow a hydrogen deficiency to be calculated (i.e. C$_n$H$_{2n+z}$ONH) and thus the degree of cyclisation. Pathways w, n and d will help establish the length of the amide chain. Thus, the MS$^2$ data should allow a significantly improved level of understanding of the structures of unknown NAs.

<table>
<thead>
<tr>
<th>Common losses $(m/z)$</th>
<th>Structure</th>
<th>Pathway</th>
<th>Fragment ion</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>NH$_3$</td>
<td>b</td>
<td>R$\equiv$C=O$^+$</td>
</tr>
<tr>
<td>18</td>
<td>H$_2$O</td>
<td>a</td>
<td>R$\equiv$C=NH$_2^+$</td>
</tr>
<tr>
<td>35</td>
<td>NH$_3$ and H$_2$O</td>
<td>c / a+e / b+f</td>
<td>R$\equiv$C=C$^+$</td>
</tr>
<tr>
<td>45</td>
<td>(base ion)</td>
<td>w</td>
<td></td>
</tr>
<tr>
<td>59</td>
<td>(base ion)</td>
<td>n / b+f+j</td>
<td></td>
</tr>
<tr>
<td>N/A</td>
<td>N/A</td>
<td>d</td>
<td>(m/z 88.1)</td>
</tr>
</tbody>
</table>

Table 4.1. Common ESI-MS$^2$ amide losses, structures, pathways and fragment ions.
4.2.3 Analysis of surrogate naphthenic amides using high performance liquid chromatography/electrospray ionisation-mass spectrometry (HPLC/ESI-MS)

The liquid chromatography separation of NAds was investigated using a 100 x 2.1 mm, 5 μm Ascentis™ RP-Amide HPLC Column (Supelco, Bellefontr, USA) and a 150 x 2.0 mm, 5 μm Gemini C18 HPLC column (Phenomenex®, UK.). Figure 4.21 shows a HPLC/ESI-MS full scan mass chromatogram (base peak plot) of the separation of surrogate NAds A to J (Table 4.2; 5 μl injection loop; ca 20 ppm per surrogate) using the Gemini column, while Figure 4.22 shows a HPLC/MS-ESI mass chromatogram (base peak plot) of the surrogates separated using the Ascentis™ column. Both columns achieved very similar separation showing resolution of all surrogates and even allowing separation of some stereoisomers (F/F', G/G' and I/J). All subsequent analyses of complex NAd mixtures reported here utilised the Gemini column chosen for wide ranging pH stability and extended column lifetime.
Figure 4.21. HPLC/ESI-DDMS mass chromatogram (base peak plot) of surrogate NAdS by gradient elution ([time/%A] 0/35-5/35-30/0; A = 0.1 % formic acid, B = 0.1 % formic acid in methanol); 150 x 2.0 mm, 5 μm Gemini C18 HPLC column. Peak assignments shown in Table 4.2.

Figure 4.22. HPLC/ESI-MS mass chromatogram (base peak plot) of surrogate NAdS by gradient elution ([time/%A] 0/35-5/35-30/0; A = 0.1 % formic acid, B = 0.1 % formic acid in methanol); 100x2.1 mm, 5 μm Ascentis™ RP-Amide HPLC column. Peak assignments shown in Table 4.2.
Table 4.2. Peak assignments for HPLC separation of surrogate NAds (Figure 4.21 and Figure 4.22).

<table>
<thead>
<tr>
<th>Peak letter</th>
<th>Surrogate</th>
<th>Structure</th>
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<tr>
<td>A</td>
<td>2-(4'-Methylcyclohexyl)acetamide</td>
<td><img src="image1" alt="Structure" /></td>
</tr>
<tr>
<td>B</td>
<td>Decahydro-2-naphthanamide</td>
<td><img src="image2" alt="Structure" /></td>
</tr>
<tr>
<td>C</td>
<td>4-Cyclohexyl-butanamide</td>
<td><img src="image3" alt="Structure" /></td>
</tr>
<tr>
<td>D</td>
<td>2,2-Dicyclohexylacetamide</td>
<td><img src="image4" alt="Structure" /></td>
</tr>
<tr>
<td>E</td>
<td>4-Pentylcyclohexylcarboxamide</td>
<td><img src="image5" alt="Structure" /></td>
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<tr>
<td>F/F'</td>
<td>cis/trans- 4-(4'-sec-butylcyclohexyl) butanamide</td>
<td><img src="image6" alt="Structure" /></td>
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<tr>
<td>G/G'</td>
<td>cis/trans- 4-(4'-n-butylcyclohexyl) butanamide</td>
<td><img src="image7" alt="Structure" /></td>
</tr>
<tr>
<td>H</td>
<td>cis-Oleicamide</td>
<td><img src="image8" alt="Structure" /></td>
</tr>
<tr>
<td>I</td>
<td>trans-Oleicamide</td>
<td><img src="image9" alt="Structure" /></td>
</tr>
<tr>
<td>J</td>
<td>Cholanamide</td>
<td><img src="image10" alt="Structure" /></td>
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4.2.4 Analysis of commercial naphthenic amides using high performance liquid chromatography/electrospray ionisation-mass spectrometry

A sample of commercially available NAs (Fluka) was converted to the amides (NAds) (Section 4.2.1) and analysed by HPLC/ESI-MS (Figure 4.23; base peak plot). The base peak chromatogram shows a degree of resolution between homologous series of NAds, with the most intense series at ca 26 - 27 min showing a molecular ion of m/z 298. Using the empirical formula for protonated NAds, $C_nH_{2n+2}ONH$ (Table 4.2), the m/z 298 series of ions can be assigned to a NAd with 19 carbons and no degrees of unsaturation (i.e. acyclic). Extracted ion series allow comparison of NAd composition between NA samples, with Figure 4.24 showing the extracted ion series for $Z = -4$ (i.e. bicyclic NAds) as an example. Although this level of molecular characterisation has previously been achieved with NAs by negative ion ESI and various GC-MS techniques (reviewed recently by Clemente and Fedorak, 2005), the use of data dependant MS² yields unique fragmentation (and hence structural) information of individual NAds (Section 4.2.6).
Figure 4.23. HPLC/ESI-MS mass chromatogram (base peak plot) of Fluka NAds by gradient elution ([time/%A] 0/35-5/35-30/0; A = 0.1 % formic acid, B = 0.1 % formic acid in methanol); 150 x 2.0 mm, 5 µm Gemini C18 HPLC column.

Figure 4.24. HPLC/ESI-MS extracted mass chromatograms from Fluka NAds (A) base peak plot, (B) m/z 196, (C) m/z 210, (D) m/z 224, (E) m/z 238 and (F) m/z 252.
Table 4.3. Expected nominal masses of protonated NAdS based on carbon and Z numbers.

<table>
<thead>
<tr>
<th>Carbon No.</th>
<th>Hydrogen deficiency (Z number)</th>
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<tr>
<td></td>
<td>0</td>
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<tr>
<td>5</td>
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<td>29</td>
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</tr>
<tr>
<td>30</td>
<td>452</td>
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4.2.5 Solid-phase extraction of naphthenic acids from crude oil

Naphthenic acids (NAs) were extracted from five crude oils of unknown provenance (personal communication LGC Ltd.) using a nonaqueous ion exchange solid-phase extraction (SPE) based on a method reported by Jones et al. (2001). Figure 4.25 shows GC-MS TIC chromatograms of 5 NA fractions (as TMS esters) collected from 5 high TAN oils, along with a procedural blank extraction. Table 4.4 shows the amount of NAs collected. The procedural blank exhibited 5 significant peaks, two of which were recovery standards and the other three contaminants (identities unknown). The 5 NA fractions showed large unresolved complex mixtures (UCMs), with exception of the 5 peaks present in the procedural blank. The amount of the NA fractions of each oil increased along with the TAN, as demonstrated in Figure 4.26. This is as expected as the TAN value is a
measurement of the amount of acid present in the oil (obtained by titrating with KOH). NAs were also extracted from a sample of Athabasca oil sands (Figure 4.27; Athabasca, Canada), from which 33.5 mg of NA fraction was collected from 1.61 g of oil (oil removed from sand by soxhlet extraction; 24h, DCM). Characterisation of the individual NAs within the UCMs was not possible by GC-MS as commonly observed (Chapter 1, Section 1.9 page 14).

Two of the NA fractions (those from the Athabasca oil sands and from the TAN 1.8 oil sample), were derivatised to NAds (Section 4.2.1) for HPLC/ESI-MS/MS analysis (Section 4.2.6).
Figure 4.25. Gas chromatograms of Crude oil SPE acid fractions (as TMS esters). (HP 5890 FID, Column, HP-1 30 m x 0.25 mm id x 0.25 μm film; oven programme; 40 – 300 °C at 10 °C min⁻¹, hold 10 min; injector 250 °C; detector 28 °C). IS = internal standard.
Table 4.4. Summary of amounts of NAs isolated by SPE from oils.

<table>
<thead>
<tr>
<th>TAN of oil</th>
<th>mg g⁻¹ oil</th>
</tr>
</thead>
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<tr>
<td>2.3</td>
<td>16.0</td>
</tr>
<tr>
<td>1.8</td>
<td>16.2</td>
</tr>
<tr>
<td>1.65</td>
<td>13.0</td>
</tr>
<tr>
<td>1.30</td>
<td>9.0</td>
</tr>
<tr>
<td>0.85</td>
<td>6.6</td>
</tr>
</tbody>
</table>

Figure 4.26. Correlation between TAN of oil and amounts of NAs isolated by SPE.

Figure 4.27. Gas chromatograms of Athabasca Oil Sands SPE NA fraction (as TMS esters). (HP 5890 FID, Column, HP-1 30 m x 0.25 mm id x 0.25 μm film; oven programme; 40 – 300 °C at 10 °C min⁻¹, hold 10 min; injector 250 °C; detector 28 °C). IS = internal standard.
4.2.6 Analysis of TAN 1.8 NAds using high performance liquid chromatography/electrospray ionisation-mass spectrometry/mass spectrometry (HPLC/ESI-MS²)

A sample of oil ‘TAN 1.8’ NAs was derivatised (NAds; Section 4.2.1) and analysed by HPLC/ESI-MS². MS² data were acquired by performing data dependent mass spectrometry. Figure 4.28 shows a full MS base peak mass chromatogram of the TAN 1.8 NAds using a gradient elution ([time/%A] 0/35-5/35-30/0; A = 0.1 % formic acid, B = 0.1 % formic acid in methanol) on a 150 x 2.0 mm, 5 μm Gemini C18 HPLC column. A peak due to the internal standard (Adamantane carboxamide; 1.8 ppm) is seen at 4.78 min, while rather poorly resolved series of peaks due to the NAds are seen at higher retention times. Extracted ion series were used to extract NAd series of differing carbon and Z numbers. Figure 4.29 shows the most abundant Z series where Z = -4 (bicyclic NAds). The product ions of MS² allowed tentative identification of NAds, following the fragmentation pathways established with MSⁿ of NAd surrogates (Section 4.2.2.9). Figure 4.30 to 4.31 show selected MS² spectra of a series of bicyclic NAds. The fragmentation of all four compounds show the characteristic [M+H]+ - 17/18 and [M+H]+ -35, as expected for NAds, and intense ions at [M+H]+ - 59. The intense ion at [M+H]+ - 59 is thought to be the cleavage of acetamide from the ring structure as observed with 2-(4'-methylcyclohexyl)acetamide (Figure 4.7). The bicyclic moiety may comprise two cyclopentyl rings, two cyclohexyl rings, or one cyclohexyl and one cyclopentyl ring, although this cannot currently be identified. Assuming both rings were cyclohexyl in the series of compounds (as was proposed by Gabryelski and Froese, 2003), conclusions can be drawn to the number of carbons additionally attached to the ring. For example, Figure 4.30 shows a compound with a molecular ion of m/z 210, therefore the loss of acetamide results in bicyclic carbocation with a C₁ group (methyl) attached to it. Figure 4.31 shows a compound with a molecular ion of m/z 224, and again with the loss of acetamide from the ring, a bicyclic carbocation is left with a C₂ unit attached, either as two methyl groups or an ethyl group. This trend continues with compounds showing molecular ions at m/z 238.
and 252 (Figure 4.32 and Figure 4.33, respectively) comprising the cyclohexyl bicyclic moiety with an acetamide group and C₃ and C₄ groups attached, respectively. These spectra indicate the length of the alkanoate moiety of the unknown NA, and with increased study, the unknown molecules may be better or even, fully characterised.

Although many other mass spectra were observed to those shown in the examples here, the \([M+H]^+ - 59\) (acetamide) ion was widely observed throughout the MS² spectra and is indicative that the ethanoate moiety is common in all NAs. Interestingly, this is constant with the findings of the biodegradation studies herein (Chapter 3) where metabolites with ethanoate chains were common place.

![Figure 4.28. MS full scan of TAN 1.8 NAds.](image)

**Figure 4.28.** MS full scan of TAN 1.8 NAds.
Figure 4.29. MS full scan (DDMS) of TAN 1.8 Nads, extracted ion series Z-4 series (196-280).

Figure 4.30. TAN 1.8 NAds MS² of parent ion m/z 210 (AA = 35 %), proposed structure inset.
Figure 4.31. TAN 1.8 NAds MS$^2$ of parent ion $m/z$ 224 (AA = 35 %), proposed structure inset.

Figure 4.32. TAN 1.8 NAds MS$^2$ of parent ion $m/z$ 238 (AA = 35 %), proposed structure inset.
Figure 4.33. TAN 1.8 NAds MS² of parent ion m/z 252 (AA = 35 %), proposed structure inset.
4.2.7 Analysis of Athabasca oil sands NAdS using high performance liquid chromatography/electrospray ionisation-mass spectrometry/mass spectrometry (HPLC/ESI-MS$^2$)

A sample of Athabasca Oil Sands was derivatised to form NAdS (Section 4.2.1) and analysed by HPLC/ESI-MS$^2$. MS$^2$ data were acquired by performing data dependent mass spectrometry (DDMS), as described in Section 4.2.6. Figure 4.34 shows a full MS base peak mass chromatogram of the Athabasca Oil Sands using a gradient elution ([time/%A] 0/35-5/35-30/0; A = 0.1 % formic acid, B = 0.1 % formic acid in methanol) on a 150 x 2.0 mm, 5 μm Gemini C18 HPLC column. The peak due to the internal standard (Adamanthane carboxamide; 1.6 ppm) is seen at 4.78 min, while little else is observed in the chromatogram. Extracted ion series were used to extract NAd series of differing carbon and Z numbers. Figure 4.35 shows the most abundant Z series where Z = -4 (bicyclic NAdS). Once again, the product ions of MS$^2$ allowed tentative identification of NAdS, following the fragmentation rules established with MS$^n$ of NAd surrogates (Section 4.2.2.9). Figure 4.36 to 4.37 show selected MS$^2$ spectra of a series of bicyclic and tricyclic NAdS. The fragmentation of all four compounds shows the characteristic [M+H]$^+$ - 17/18 and [M+H]$^+$ -35, as expected for NAdS, and intense ions at [M+H]$^+$ - 59. The intense ion at [M+H]$^+$ - 59 is thought to be the cleavage of acetamide from the ring structure as observed with 2-(4'-methycyclohexyl)acetamide (Figure 4.7). As before, assuming all ring moieties were cyclohexyl in the series of compounds, conclusions can be drawn to the number of carbons additionally attached to the ring. For example, Figure 4.36 and Figure 4.37 show spectra of compounds with molecular ions of m/z 238 and 252 (Z = -4), both showing a loss of acetamide indicating that 3 and 4, respectively, additional carbons must be attached to the bicyclic moiety. Similarly Figure 4.38 and Figure 4.39 show spectra of compounds with molecular ions of m/z 250 and 264 (Z = -6), and again both show the loss of acetamide from the ring, but the higher molecular weight of the second means an additional carbon must be attached to the tricyclic moiety. As with the oil ‘TAN 1.8’
NAd's, these spectra show the length of the alkanoic moiety of the unknown NAd's. Once again the \([\text{M}+\text{H}]^+ - 59\) (acetamide) ion was widely observed throughout the MS$^2$ spectra.

Figure 4.34. MS full scan (DDMS) of Athabasca Oil Sands NAd's.
Figure 4.35. MS full scan (DDMS) of Athabasca Oil Sands Nads, extracted ion series m/z 196 – 280.

Figure 4.36. MS² of parent ion m/z 238 (AA = 35 %), proposed structure inset.
Figure 4.37. MS² of parent ion m/z 252 (AA = 35 %), proposed structure inset.

Figure 4.38. MS² of parent ion m/z 250 (AA = 35 %), proposed structure inset.
Figure 4.39. MS² of parent ion m/z 264 (AA = 35 %), proposed structure inset.
4.3 Conclusion

The derivatisation of NA surrogates to NAds was achieved in quantitative yields. The derivatisation and analysis by electrospray ionisation ion trap multi-stage mass spectrometry of surrogate NAds in positive ion mode resulted in an improvement in response of over 2 orders of magnitude compared to that of NA surrogates in negative ion mode. Limits of detection (LOD) were not established herein and need to be established in future work. The improved response following derivatisation to amides allowed multistage MS (MS\textsuperscript{4}) experiments, providing detailed fragmentation pathways for individual NAds to be established.

Following SPE extraction, two real NAs mixtures (isolated from an oil and a tar sands sample) were derivatised to NAds and separated by a HPLC method optimised for mixtures of synthetic surrogate NAds. Data dependent mass spectrometry provided unique MS\textsuperscript{2} spectra of individual NAds, from which identification of the alkanoic chain length was possible. Tentative characterisation of the structure of the unknown NA was made, and series of NAs tentatively identified. The predominant alkanoic chain length was found to be ethanoate, a finding that is consistent with biodegradation studies where metabolite end points contained an ethanoate moiety (Chapter 3). This is consistent with widespread beliefs that NAs are recalcitrant mixtures resulting from the biodegradation of crude oil hydrocarbons (e.g. Aitken et al. 2004).

The level of characterisation of individual NAs from complex mixtures by ESI-MS, once derivatised to NAds, is greater than reported previously. This method promises to be particularly beneficial for future studies of NA toxicity, bioremediation and corrosive properties.

Future work should investigate the use of HPLC/ESI-MS\textsuperscript{n} for quantitative analysis of NAs.
Chapter 5 Experimental details

5.1 General laboratory procedures

All glassware was soaked in Decon 90™ (overnight), rinsed thoroughly with hot tap water (x3), then rinsed with distilled water (x3), and oven dried (120 °C; overnight). Glassware was covered with aluminium foil during storage, and solvent rinsed (x3) with clean solvent immediately prior to use.

All laboratory and gas chromatography solvents were HPLC or glass distilled grade (Rathburn Chemical Ltd, Walkerburn, U.K.). Solvent purity was routinely monitored by gas chromatography (GC) analysis (0.5 µL aliquot) of a 100 mL sample that had been rotary evaporated to around 1 mL (Buchi, 40°C), transferred to a vial and evaporated under nitrogen to approximately 0.5 mL.

Electrospray ionisation-mass spectrometry (ESI-MS°) solvents were Chromasolv® LC-MS grade (Riedel-de Haën, Sigma Aldrich, UK). Solvent purity was constantly monitored through blank analyses.

5.2 Instrument details

5.2.1 Gas chromatography flame ionisation detection (GC-FID)

Separations of non-polar analytes were performed with a HP5890 Series II gas chromatograph (Hewlett-Packard, USA) fitted with a flame ionization detector (FID) and a HP7673 autosampler. General GC-FID conditions were: 1 µl splitless injection; injector temperature 250 °C; column Agilent Ultra-I (12.5 m x 0.20 mm x 0.33 µm) GC programme, 40 - 300 °C at 5 °C min⁻¹, hold 10 min; detector temperature 300 °C. Data were recorded using Turbo Chrom Navigator (Version 6.1.1; Perkin Elmer, UK).

5.2.2 Gas chromatography-mass spectrometry (GC-MS)

Separations of non-polar analytes were performed using a HP5890 Series II gas chromatograph (Hewlett-Packard, USA) fitted with a HP5970 Series Mass Selective Detector and HP7673 autosampler. General GC-MS conditions were: 1 µl splitless
injection; injector temperature 250 °C; column Agilent Ultra-1 (12.5 m x 0.20 mm x 0.33 μm) GC programme, 40 - 300 °C at 5 °C min⁻¹, hold 10 min; head pressure 40 kPa; electron multiplier, 1600-2200 V. Data was collected using HP Chemstation (Version C.03.00.00) software.

5.2.3 Nuclear magnetic resonance (NMR) spectroscopy

1H, 13C and DEPT nuclear magnetic resonance spectroscopy was carried out using a Jeol EX-270 FT-NMR spectrometer. Samples were dissolved in deuterated chloroform (CDCl₃) or water (CD₃OD).

5.2.4 Infrared (IR) spectroscopy

Infrared spectroscopy was carried out using a Bruker FT-IR IFS66 spectrometer (Bruker Inc, USA) and data acquired using OPUS 2.0 Optics software (Bruker Inc, USA). Samples were prepared as liquid films (NaCl discs) or pellets (KBr discs).

5.2.5 Electrospray ionisation-multistage mass spectrometry (ESI-MS")

Mass spectrometry analysis was carried out using a Finnigan Mat LCQ™ (ThermoFinnigan San Jose, CA, USA.) bench top mass spectrometer fitted with an electrospray interface. Data were acquired and processed with Xcalibur 1.0 spl software. General ESI-MS" conditions were: source voltage, (±) 4.5 kV; capillary voltage, (±) 0-50 kV (auto tune function on ion of interest); capillary temperature, 200 °C; nitrogen sheath gas flow rate, 40 (arbitrary units).

5.3 Synthesis of surrogate naphthenic acids

4-(4'-n-butylicyclohexyl)butanoic acid (n-BCHBA), 4-(4'-iso-butylicyclohexyl)butanoic acid (iso-BCHBA), 4-(4'-sec-butylicyclohexyl)butanoic acid (sec-BCHBA) and 4-(4'-tert-butylicyclohexyl)butanoic acid (tert-BCHBA) were synthesised using methods developed by Wraige (1997), Sturt (2000) and Smith (2002). Figure 5.1 illustrates the synthetic scheme, where R represents n-, iso-, sec- or tert-buty. From this point forward, full details
will be described only for the synthesis of 4-(4'-iso-butylcyclohexyl)butanoic acid as an example of the synthesis of all isomers. The only difference in the synthesis of the other isomers was that of the starting material for the Friedel-Crafts reaction (i.e. n-butylbenzene, sec-butylbenzene and tert-butylbenzene).

![Chemical structure of 4-(4'-iso-butylcyclohexyl)butanoic acid](image)

**Figure 5.1.** Scheme for the preparation of 4-(4'-R-butylcyclohexyl)butanoic acid (R = n-, iso-, sec- or tert-butyl).

### 5.3.1 Preparation of 4-(4'-iso-butylphenyl)-4-oxo-butanoic acid

![Chemical structure of 4-(4'-iso-butylphenyl)-4-oxo-butanoic acid](image)

**Figure 5.2.** Reaction scheme for the synthesis of 4-(4'-iso-butylphenyl)-4-oxobutanoic acid (I).
4-(4'-iso-butylphenyl)-4-oxo-butanoic acid (I) was synthesised using Friedel-Crafts acylation of iso-butylbenzene (Figure 5.2). Details of Friedel-Crafts reactions can be found in March (1985) and Vogel (1989).

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\text{4-(4'-iso-butylphenyl)-4-oxo-butanoic acid (I) was synthesised using Friedel-Crafts acylation of iso-butylbenzene (Figure 5.2). Details of Friedel-Crafts reactions can be found in March (1985) and Vogel (1989).}
\]

**Figure 5.3.** Apparatus for Friedel-Crafts acylation.

The apparatus was assembled as shown above (Figure 5.3). Typically, succinic anhydride (12 g, 0.12 mol), iso-butylbenzene (0.1 mol, 13.4 g) and 1,1,2,2-tetrachloroethane (100 mL) were placed in a three-neck 250 mL round bottomed flask (rbf). The mixture was heated gently using an electric heating mantle (80 °C), with stirring by mechanical stirrer to dissolve the succinic anhydride, producing a clear yellow liquid. On cooling, 32 g (0.24 mol) of finely ground aluminium chloride was added in small portions over a 30 minute period. This was left stirring for 1 hour during which time the mixture turned yellow, orange, red and finally to dark red and became more viscous. After this time the contents of the flask were poured into a 400 mL beaker half filled with ice and rinsed with distilled
water. Concentrated HCl (~50 mL) was added until no more dark red material was apparent and the mixture was a banana colour. This mixture was then transferred to a 500 mL rbf ready for steam distillation.

The mixture was distilled (2 h) with addition of water as necessary to keep the level in the flask at around 300 mL. At this time the majority of the tetrachloroethane (~95%) had been collected. Once cooled, Na₂CO₃ (1M) was added until all the HCl was neutralised, indicated by the cessation of effervescence and pH paper measurements. The alkaline mixture was steam distilled again until all the tetrachloroethane was recovered.

The distilled mixture was placed in a beaker on a hotplate, further Na₂CO₃ (1M) was added to dissolve the product, and 1 g decolourising carbon was added. Precipitated alumina was removed by filtration using a heated filter funnel and the filtrate left to cool. This was slowly acidified with concentrated HCl which resulted in the formation of a creamy precipitate upon neutralisation. The product was obtained by vacuum filtration, washing with water to remove any HCl, and left to dry in a drying cabinet (40 °C) overnight.

5.3.2 Preparation of 4-(4'-iso-butylphenyl)butanoic acid

![Reaction scheme for the synthesis of 4-(4'-iso-butylphenyl)butanoic acid (II).](image)

4-(4'-iso-butylphenyl)-4-oxobutanoic acid (II) was converted into 4-(4'-iso-butylphenyl)butanoic acid via a Huang-Minlon modification of a Wolff-Kischner reduction (Huang-Minlon, 1946; Figure 5.4).

KOH (6 g, 0.1 mol) was dissolved in diethylene glycol (40 mL) by gentle heating. Hydrazine hydrate (3.2 mL, 0.0663 mol) and 4-(4'-iso-butylphenyl)-4-oxo-butanoic acid (11.5 g, 0.044 mol) were added and the mixture refluxed (1 h, 130 °C) to form the
hydrazone. Once the hydrazone was formed the aqueous material was distilled off and collected in order to reach a high enough temperature for the decomposition of the hydrazone. This was achieved by reflux (4 h, 230 °C). The resulting material was left overnight to cool and then poured into a 200 mL beaker half filled with ice and acidified with concentrated HCl to Congo Red whilst stirring with a glass rod. Upon neutralisation a brown sticky lump was formed which was extracted with diethyl ether (100 mL), water washed (3 x 50 mL) and dried over anhydrous Na₂SO₄ (~5 g). The resultant extract was treated with decolourising carbon (~1 g), followed by hot filtration and rotary evaporation.

5.3.3 Hydrogenation of 4-(4'-iso-butylphenyl)butanoic acid

![Reaction scheme for hydrogenation of 4-(4'-iso-butylphenyl)butanoic acid](image)

**Figure 5.5.** Reaction scheme for hydrogenation of 4-(4'-iso-butylphenyl)butanoic acid (II) to the surrogate naphthenic acid 4-(4'-iso-butylcyclohexyl)butanoic acid (iso-BCHBA; III).

Hydrogenation of 4-(4'-iso-butylphenyl)butanoic acid was achieved using a method adapted from Dauben and Hoerger (1951). A mixture of 4-(4'-iso-butylphenyl)butanoic acid (200 mg) in glacial acetic acid (10 mL) and platinum oxide (100 mg; 99.5%, Aldrich, UK) was hydrogenated at ca. 8 psi at room temperature for varying times. The pressure was regulated through a HPLC regulator with the apparatus set up as shown below (Figure 5.6). All joints were sealed externally with parafilm and quick-fit clips. The catalyst was removed by filtration and the acetic acid by rotary evaporation.
5.3.4 Synthesis of 4-(4'-iso-butylphenyl)-4-methylbutanoic acid

4-(4'-iso-butylphenyl)-4-methylbutanoic acid (iso-BCHMBA) was synthesised using the method described for iso-BCHBA (Sections 5.3.1 - 5.3.3) with the exception that γ-valerolactone was used instead of succinic anhydride (Section 5.3.1; Figure 5.7). Because the product of the Friedel-Crafts acylation was not a keto acid, the Wolff-Kischner reduction was not necessary. However, the product yielded three isomers which required fractionation by reverse-phase HPLC (Section 5.3.5) and characterisation prior to hydrogenation.
iso-butylbenzene + γ-valerolactone

$$\text{ACl}_3$$

$$\text{H}_2, \text{PtO}_2$$

(+ o- and m-isomers)

Figure 5.7. Scheme for the preparation of 4-(4'-iso-butylecyclohexyl)-4-methylbutanoic acid. (4-(4'-iso-butylecyclohexyl) isomer shown for convenience, but 2' and 3' isomers were also produced).

5.3.5 Reverse-phase high performance liquid chromatography (HPLC) fractionation

Reverse–phase HPLC fractionation was performed using an Agilent 1100 system with an integrated automatic fraction collector. Separation was achieved using a 150 x 10.0 mm, 5 μm Gemini C18 HPLC column (Phenomenex®, UK) with an isocratic mobile phase (methanol:H₂O:Formic acid; 75:25:0.1; v/v/v) at a flow rate of 3.0 mL min⁻¹. UV-Vis detection was performed at 206 nm. It was determined experimentally that 10 mg of isomeric intermediates on-column was the maximum amount per run that allowed both good separation and recovery of fractions. Fractions were collected corresponding to UV absorption minima and analysed by GC-MS. Fractions were cut at 15.1-16.2 min (Figure 5.8; Fraction 1; Isomer 1), 18.3-19.4 min (Fraction 2; Isomer 2) and 20.0-21.2 min (Fraction 3; Isomer 3).
Figure 5.8. HPLC UV chromatogram (206 nm) showing three 4-(iso-butylcyclohexyl)-4-methylbutanoic acid isomers with fraction collection times. (Agilent 1100 system; 150 x 10.0 mm, 5 µm Gemini C18 HPLC column; mobile phase, methanol:H2O:Formic acid; 75:25:0.1 (v/v/v); 3 mL min⁻¹).

5.4 Acute oyster embryo larval development test

The Environment Agency Ecotox 03 Oyster Embryo-Larval Development Test procedure was followed.

Instant Ocean (Sigma-Aldrich; UK) was used as reference sea water. The solution (33.3 g L⁻¹) was stirred and oxygenated over night, and physical parameters measured: Dissolved O₂ = 5.18 mg L⁻¹; Salinity = 34.5%; pH = 8.30.

5.4.1 Strip spawning

Oyster embryos were prepared by strip spawning each of two adult males and two adult females.

The oysters (*Crassostrea gigas*) were opened by inserting an oyster knife in the flat edge of the oyster and cutting the adductor muscle to avoid damaging the gonads. The body cavity of each oyster was rinsed thoroughly with reference sea water to minimise contamination of the gametes. Sperm and eggs were removed by making a small incision with a scalpel into the gonads and carefully removing sperm or eggs with a glass pipette.
The two sperm samples from the separate males were pipetted (glass Pasteur pipettes) into separate beakers with reference sea water (~100 mL). The beakers were covered and left to stand for at least 10 minutes in an incubator (24 ±2 °C) to allow activation of the sperm. Sperm were then observed under a microscope and the sample with the highest motility was selected for subsequent use in the toxicity assay.

Eggs obtained from the females were placed into two separate beakers and incubated (24 ±2 °C) whilst being magnetically stirred to ensure a homogenous suspension. The density of each of the batches of eggs was measured immediately by counting the number of eggs in 10μl using a microscope (triplicate). The egg suspension was diluted to give a density of 600 egg mL⁻¹.

5.4.2 Fertilisation

A small sample of each egg suspension was pipetted onto a clean slide using a Pasteur pipette. The eggs were observed under a microscope to ensure that the majority of the eggs were spherical, indicating that they remained viable, as opposed to ‘tailed’ or ‘pear’ shaped indicative of non-viable eggs (Figure 5.9).

![Figure 5.9. Light micrographs of female oyster (C. gigas) eggs (a) immature ('pear' shaped, non-viable), (b) mature (round, viable) and (c) fertilised eggs.](image)

Eggs were fertilised by adding sperm sample (6 mL) and the gametes were then left in the dark (24 ±2 °C; 1 hr) before checking the fertilisation rate. The gametes were incubated until fertilisation rate reached 95% (approximately 1.5 hours).
5.4.3 Preparation of test solutions for initial screening study

Fertilised oyster eggs were inoculated with three surrogate NAs separately (sec-BCHBA, DHNA and cholanic acid; CA) at concentrations of 0.001, 0.01, 0.1 and 1.0 mg L$^{-1}$ from stock solutions (0.002, 0.02, 0.2 and 2.0 mg L$^{-1}$, respectively). Methanol was used as a carrier solvent for the surrogate NAs (0.5 mL). Stock solution (25 mL) was added to the fertilised egg solutions (25 mL; 200 fertilised eggs mL$^{-1}$) in beakers (100 mL) to give final concentrations of 0.001, 0.01, 0.1 and 1.0 mg L$^{-1}$ of surrogate NAs and 100 fertilised eggs mL$^{-1}$. Each surrogate was run individually at the four concentrations in duplicate (i.e. 24 beakers). Sea water controls (sea water and fertilised eggs), solvent controls (sea water, 0.5 mL methanol and fertilised eggs) and positive controls (sea water, zinc 0.1 mg L$^{-1}$) were carried out in triplicate. The assay was initiated at the point of the inoculation of fertilised eggs with surrogate NAs.

5.4.4 Preparation of test solutions for detailed study

Test solutions for the detailed study were prepared in the same manner as described in Section 5.4.3 except that fertilised oyster eggs were incubated with n-BCHBA, iso-BCHBA, sec-BCHBA and tert-BCHBA at the same four concentrations (i.e. 48 beakers). Controls and assay initiation were as described in Section 5.4.3.

5.4.5 Termination of the toxicity assay

Embryos were fixed (Baker’s Formalin, 1 mL) for subsequent assessment following 24 h exposure to the surrogate NAs. Baker’s formalin was made up as follows: calcium chloride (1.0 g), cadmium chloride (1.0 g), and concentrated formalin (10 mL), accurately diluted to a final volume of 100 mL with distilled water.

5.4.6 Assessment of oyster embryo development

After fixing, embryos were left to settle to the bottom of the beaker and the majority of the sea water was discarded. 100 μL of embryo solution containing a nominal 100-300 embryos was pipetted onto a welled slide and the normal and abnormal embryos were
counted under magnification using a light microscope and the classification system of His et al. (1997; Figure 5.10).

![Figure 5.10](image)

**Figure 5.10.** Abnormalities observed in *Crassostrea gigas* D larvae according to His et al. (1997). (a) unsegmented eggs, normal or abnormal blastula, gastrula and trophophore, (b) normal larva, (c) convex hinge, (d) indented shell margin, (e) incomplete shell, (f) protruded mantle.

### 5.5 Mixed culture biodegradation studies

Samples of marine sediment (50g) were obtained in January 2003, by Dr C. Whitby (University of Exeter), from locations in the South-West Region of the UK including Devonport, Avonmouth and Portishead (Figure 5.11). These sites had been subjected to periodic previous exposures to hydrocarbon contamination for several years. Samples were also obtained from Ons Island (North West coast of Spain) and a sample of oil well drill cuttings (supplied by MI Drilling fluids™ but of unknown origin). The sediments were stored at 4°C within 2h of sampling and subsamples were also removed and stored at -20°C. Starter cultures were prepared by inoculating approximately 1g sediment obtained from each environmental sample into a minimal medium (50 mL) containing (g L\(^{-1}\) of distilled water): MgSO\(_4\), 0.2; (NH\(_4\))\(_2\)SO\(_4\), 0.5; KH\(_2\)PO\(_4\), 0.5; K\(_2\)HPO\(_4\), 1.5; NaOH, 0.02; Na\(_2\)EDTA, 0.12; ZnSO\(_4\), 0.004; CuSO\(_4\), 0.001; Na\(_2\)SO\(_4\), 0.0001; Na\(_2\)MoO\(_4\), 0.001; MnSO\(_4\), 0.0004; CoCl\(_2\), 0.0001 and the medium adjusted to pH 7.0.
5.5.1 Initial biodegradation screening study

Initial screening was carried out using all five cultures to test which culture was best adapted to degrading NAs. All glassware was sterilised by autoclave (121 °C, 20 min).

5.5.2 Reagents and inoculum

Sterilised medium (50 mL; minimal media Section 5.5) was added to each of 16 culture flasks (100 mL). This number of flasks allowed for three compounds to be incubated with each culture for a single time point with one abiotic control. The three surrogate NAs used were n-BCHBA, sec-BCHBA and decahydro-2-naphthoic acid (2-naphthoic acid hydrogenated in-house; purity >90%; DHNA). Surrogates were dissolved in methanol (HPLC grade, Rathburn Chemicals Ltd., Scotland; 4 mg mL⁻¹) and injected (50 μL, 0.2 mg) into each culture flask (and also into three separate GC vials as a reference of 100 % recovery) to which bacterial inoculum (1 mL) was added and flasks were capped with a Teflon seal. Incubation was performed aerobically in an incubator (25 °C). The abiotic
control contained the media and the surrogate NAs, but no bacterial inoculum and was incubated under the same conditions to monitor any abiological losses.

5.5.3 Measurement of bacterial viability
At the point of sampling, sterile techniques were used to streak a solution sample from each culture flask onto an agar nutrient plate. This was incubated (25 °C; 24 h) and any bacterial growth was taken as evidence of viability.

5.5.4 Surrogate NA extraction
1-Adamantane carboxylic acid (99 %; Aldrich, UK) and n-eicosane (n-C₂₀; 99 %; Aldrich, UK) were used as internal standards during extraction and analysis in order to monitor losses of the surrogate NAs at each stage. Internal standards were dissolved in methanol (1 mg mL⁻¹) and injected (50 μL, 0.05 mg) into each culture flask prior to extraction and also into three separate GC vials as a reference of 100 % recovery. Aqueous sample (25 mL) was transferred using a glass pipette into a glass Quickfit boiling tube (50 mL), acidified to pH 2 (conc. HCl) and extracted with ethyl acetate (3 x 15 mL; HPLC grade, Rathburn Chemicals Ltd., Scotland). Emulsions were removed by centrifugation (2500 rpm, 5 min).

Extracts were combined, dried (Na₂SO₄; overnight) and evaporated to near-dryness (Buchi, 40 °C), transferred to GC vials and gently blown down to dryness (N₂). BSTFA derivatising agent (20 μL) was added to samples and heated (70 °C for ca. 20 min) before making up samples in dichloromethane (1 mL; HPLC grade, Rathburn Chemicals Ltd., Scotland) ready for analysis using GC-MS (Section 5.2.2). Quantification was made by comparing analyte peak areas to those of the reference samples and adjusting by internal standard recoveries.

5.6 Devonport consortium mixed culture biodegradation study

5.6.1 Reagents and inoculum
Sterilised medium (25 mL; minimal media Section 5.5) was added to 126 culture flasks (50 mL). This number of flasks allowed for four surrogate NAs (n-BCHBA, iso-BCHBA, sec-
BCHBA and tert-BCHBA) to be inoculated in triplicate for seven time points, individual abiotic controls for each surrogate in triplicate for three time points, and procedural blanks in triplicate at two time points. Surrogate compounds were each dissolved in methanol (1 mg mL\(^{-1}\)) and injected (100 \(\mu\)L; 0.1 mg) into each culture flask and also into separate GC vials, in triplicate, as a reference of 100 % recovery. Lastly bacterial inoculum (0.5 mL) was added and flasks capped with Teflon seals. Incubation was performed aerobically (25 °C). The abiotic control contained the media and the surrogate NAs but no bacterial inoculum and the procedural blanks contained the media and surrogate NAs but no bacteria. Both were incubated under the same conditions to monitor any abiological losses. Bacterial viability was measured at each time point for each sample (Section 5.5.3).

### 5.6.2 Surrogate NA extraction

1-Adamantanecarboxylic acid was used as an internal standard during extraction and analysis to monitor any losses of the surrogate NAs. Internal standards were dissolved in methanol (1 mg mL\(^{-1}\)) and injected (50 \(\mu\)L; 0.05 mg) into each culture flask prior to extraction and also into three separate GC vials as a reference of 100 % recovery. Once the internal standard had been added aqueous samples were transferred to centrifuge tubes (50 mL) and centrifuged (8500 rpm, 5 minutes). The aqueous sample was carefully removed and replaced into the culture flasks leaving approximately 2 mL of aqueous sample and a bacterial pellet in the centrifuge tube. Bacteria were resuspended (Vortexer) and transferred to a microcentrifuge tube (2 mL) and centrifuged (13000 rpm, 10 minutes). Aqueous sample was again carefully removed and replaced into the original culture flask. The microcentrifuge tube containing the bacterial pellet was then frozen awaiting DNA profiling. The aqueous sample was transferred into a glass Quickfit boiling tube (50 mL), acidified to pH 2 (conc. HCl) and extracted with ethyl acetate (3 x 15 mL). Emulsions were removed by centrifugation (2500 rpm, 5 min). Extracts were combined, dried (Na\(_2\)SO\(_4\); overnight) and evaporated to near-dryness (Buchi, 40 °C), transferred to GC vials and gently blown down to dryness (\(N_2\)). BSTFA (20 \(\mu\)L) was added to each sample and
samples heated (70 °C; ca. 20 min). Samples were then made up in DCM (1 mL) ready for analysis by GC (Section 5.2.1) for quantification, and then selected samples were analysed by GC-MS (Section 5.2.2) for qualitative information.

5.7 Amide derivatisation of naphthenic acids (NAs)

Amides were prepared by treating a solution of naphthenic acid (1.0 g, 3.55 mmol, 1.0 equiv) in CH₂Cl₂ (8.9 mL, 0.4 M) with oxalyl chloride (5.32 mL, 2.0 M solution in CH₂Cl₂, 10.64 mmol, 3.0 equiv) dropwise at 0 °C. The reaction mixture was stirred at 25 °C for 4 h. The mixture was then concentrated under reduced pressure, cooled to 0 °C and treated with saturated aqueous NH₄OH (2.0 mL). The reaction mixture was then partitioned between ethyl acetate (100 mL) and H₂O (100 mL). The organic layer was removed, dried (Na₂SO₄) and concentrated under reduced pressure. Infrared spectroscopy was used to confirm that conversion from a carboxylic acid to an amide had occurred.

5.8 Infusion electrospray ionisation-multistage mass spectrometry (ESI-MS²) of surrogate naphthenic amides (NAds)

Mass spectrometry analysis was carried out using a Finnigan Mat LCQ™ (ThermoFinnigan San Jose, CA, USA) bench top mass spectrometer fitted with an electrospray interface. Data were acquired and processed with Xcalibur 1.0 spl software. Infusion of surrogates was carried using a built in syringe pump with a Hamilton 1725N (250 µL) syringe (Reno CA, USA). Analytes were infused at 3.0 µL min⁻¹ (Methanol;Water;Formic Acid; 75:25: v/v), with 0.1 % formic acid modifier added to promote protonation of amides and 0.1 % ammonia modifier added to promote deprotonation of acids. Source voltage, (±) 4.5 kV; capillary voltage, (±) 0-50 kV (auto tune function on ion of interest); capillary temperature, 200 °C; nitrogen sheath gas flow rate, 40 (arbitrary units). The auto-tune function was applied to precursor ions to optimise the instrument prior to analysis of individual surrogates. Positive and negative ion (±) MS² analysis of selected ions was performed in the ion trap by collision-induced dissociation
(CID) with helium damping gas. MS^n ion isolation widths, relative activation amplitudes and activation Qs were optimised to obtain high response and stability of the base peak fragment ion. High-resolution ZoomScan™ (ZS) spectra for all ions of interest were recorded. All spectral data were recorded and averaged over a one minute acquisition time.

5.9 Solid-phase extraction (SPE) procedure for the extraction of naphthenic acids (NAs) from crude oil

Naphthenic acids (NAs) were extracted from crude oils using a non-aqueous ion exchange solid-phase extraction (SPE) method reported by Jones et al., (2001). Briefly, a SAX quaternary amine (10 g) SPE ion exchange column (International Sorbent Technology) was conditioned with 30-40 mL of n-hexane. The oil sample (1-2 g) was spiked with 1-adamantanecarboxylic acid, and 5β-cholanic acid as recovery standards (at approximate concentrations of 75 and 50 μg·g⁻¹ of oil, respectively) and then pipetted onto the column. After the sample had adsorbed onto the column, interferents were removed by eluting with 80 mL n-hexane followed by 80 mL of dichloromethane and then the residual solvent was carefully removed by an air-flush. The acid fraction was then eluted with 55 mL of diethyl ether containing 2 % (v/v) formic acid. Solvent was removed under reduced pressure.

5.10 High performance liquid chromatography/electrospray ionisation-multistage mass spectrometry (HPLC/ESI-MS^n)

High performance liquid chromatography (HPLC) was carried out using a P580A binary pump (Dionex-Softron GmbH, Germering, Germany). Sample injections (5 μL) were made into a 200 μL·min⁻¹ flow rate. HPLC columns investigated were an 100 x 2.1 mm, 5 μm Ascentis™ RP-Amide HPLC Column (Supelco, Bellefont, USA) and 150 x 2.0 mm, 5 μm Gemini C18 HPLC column (Phenomenex®, UK.). For both columns, the optimum solvent conditions were found to require a gradient mix of water (A) and methanol (B), both containing formic acid (0.1 %). The gradient ([time/%A] 0/35-5/35-30/0) allowed good (i.e. separation in <30 min) retention and resolution of compounds with symmetrical peaks with low tailing.
Mass spectrometry was carried out using a Finnigan Mat LCQ™ (ThermoFinnigan San Jose, CA, USA.) bench top mass spectrometer fitted with an electrospray interface. Data were acquired and processed with Xcalibur 1.0 spl software. Instrument parameters were as follows; source voltage +4.5 kV; capillary voltage + 20 V; capillary temperature, 220 °C; nitrogen sheath gas flow rate, 60 (arbitrary units); and nitrogen auxiliary gas 20 (arbitrary units). Data dependent mass spectrometry acquisition is a function of the Xcalibur software that allows MS/MS experiments selecting the MS\textsuperscript{1} base ion to be the MS\textsuperscript{2} precursor ion for the subsequent scan before returning back to MS\textsuperscript{1}. This function was used to gain MS\textsuperscript{2} for NAds in complex mixtures. The activation amplitude was optimised to 35 %. Extracted ion chromatograms (EIC) were extracted from the full scan; total ion chromatograms (TIC) selected to $m/z$ 0.1 and an isolation width of $m/z$ 1.
Chapter 6 Conclusions and suggestions for further work

6.1 Conclusions

Naphthenic acids (NA s) are a naturally occurring complex mixture of alkyl substituted alicyclic carboxylic acids fitting the formula \( \text{C}_n\text{H}_{2m-z}\text{O}_2 \) which produce a gas chromatographic unresolved complex mixture (UCM) or 'hump' in much the same way as other petroleum hydrocarbons mixtures \( \text{e.g.} \) Dzidic et al., 1988; Gough and Rowland, 1990). The complexity of NA UCMs is due to the presence of hundreds, and more likely thousands, of isomeric compounds which to date have not been individually identified or quantified (Clemente and Fedorak, 2005). The toxicity of NAs to a wide variety of organisms including mammals, fish and zooplankton, at aqueous concentrations as low as 100 ppb has been shown (Clemente and Fedorak, 2005; Quargraine et al., 2005a). Although biodegradation has been shown to initially reduce the toxicity of NA mixtures the more recalcitrant, but less toxic, components remain an environmental concern (Herman et al., 1994; Clemente et al., 1994). NAs are of further concern to the oil refinery industry where both their corrosive properties and unwanted deposition in pipelines (as naphthenates) result in costly production down time.

The specific objectives of the current study were:

- To synthesise a suite of well characterised, pure, alicyclic surrogate NAs containing \textit{both} alkyl and alkanoate substituents. These are not commercially available.

- To test the toxicity of the synthetic surrogate NAs using an oyster embryo development test to establish whether the measured toxicities were comparable to those of 'real' NAs and thus determine the validity of the surrogates as model NAs.

- To monitor the biodegradation of the synthetic NAs using mixed bacterial cultures to determine the potential for bioremediation.
To develop a suitable derivatisation technique to enable electrospray ionisation MS<sup>a</sup> characterisation of individual NAAs at a molecular level.

Four isomeric monocyclic surrogate NAAs, 4-(4'-iso-butylocyclohexyl)butanoic acid (iso-BCHBA), 4-(4'-n-butylocyclohexyl)butanoic acid (n-BCHBA), 4-(4'-sec-butylocyclohexyl)butanoic acid (sec-BCHBA) and 4-(4'-tert-butylocyclohexyl)butanoic acid ( tert-BCHBA), possessing both alkyl and alkanoate moieties were synthesised in good yield (~200 mg) and purity (>94% by GC).

An initial toxicity study in which oyster (Crassostrea gigas) embryos were inoculated with sec-BCHBA, n-BCHBA, decahydro-2-naphthoic acid (a bicyclic NA) and cholic acid (a polycyclic NA) showed the two monocyclic NAAs to be less toxic than the bicyclic NA which in turn was less toxic than the polycyclic NA. The subsequent detailed study of the synthetic isomeric series, iso-, n-, sec- and tert-BCHBA, demonstrated a quantitative structure-activity relationship (QSAR) between the degree of branching in the alkyl chain and the level of abnormality observed in the developing oyster embryos. The surrogate with the least branching in the alkyl chain exhibited the greatest toxic effect (n-BCHBA EC50 0.11 mg L<sup>-1</sup>) whilst the more branched surrogates exhibited less toxic effect ( tert-BCHBA EC50 0.49 mg L<sup>-1</sup>; iso-BCHBA EC50 0.21 mg L<sup>-1</sup>; tert-BCHBA EC50 0.33 mg L<sup>-1</sup>). The QSAR has been attributed to the aqueous solubility of the surrogates (estimated using KowWin, EPI suite) where increasing water solubility decreases the toxic effect. The EC50 values obtained were comparable to those reported in previous studies and were only slightly higher than those tolerated by zooplankton (0.15 mg L<sup>-1</sup>; Dokholyan and Magomedov, 1984) but significantly lower than those required to produce a 50% mortality in two month old chub salmon (25 mg L<sup>-1</sup>; Dokholyan and Magomedov, 1984).

An initial biodegradation experiment showed that all five environmental bacterial consortia tested readily degraded sec-BCHBA and n-BCHBA (>95% degradation in 30 days), whereas decahydro-2-naphthoic proved to be far more recalcitrant (<25% degradation in
30 days). The subsequent detailed experiment using only the Devonport consortium showed significant differences in biodegradation rates for the four synthetic isomeric monocyclic surrogate NAs, iso-, n-, sec- and tert-BCHBA. n-BCHBA was the most readily degraded with 97% degradation by day 9, and tert-BCHBA the most resistant with only 2% degradation by day 30. iso-BCHBA and sec-BCHBA were 77% and 53% degraded after 30 days, respectively. The degradation of the monocyclic surrogate NAs coincided with the production of a major metabolite consistent with β-oxidation of the alkanoate chain. The metabolites were thought to be the ethanoate acid analogs of the BCHBA isomers, which themselves resisted further degradation. The degradation via β-oxidation was confirmed by the synthesis and subsequent biodegradation study of iso-BCHMBA, where the presence of a methyl branch in the alkanoate chain halted the biodegradation.

The results of the toxicity and biodegradation studies indicate a link between the toxicity and biodegradation of the surrogates and their structures. It is proposed that the degree of toxicity and resistance to biodegradation are linked to the structures of the surrogates in a QSAR affected by their hydrophobicity, which thus determines their bioavailability. This link is consistent with previous studies where initial biodegradation of NAs decreased the toxicity (Herman et al. 1994). However, of important note is that although the present study shows the most toxic surrogate (n-BCHBA) to be readily degraded, the most recalcitrant surrogate (tert-BCHBA) still exhibited a toxic effect of the same order of magnitude. Thus, bioreistant NAs will also be expected to be toxic. The biodegradation studies indicate that the bioremediation of recalcitrant NAs is reliant upon finding bacteria that will degrade the less bioavailable (e.g. tert-butyl substituted) NAs with methyl branching in the alkanoate moiety.

The unique derivatisation of NAs to naphthenic amides (NAds) was achieved in quantitative yields. The resulting NAds allowed for an improvement in detection of over two orders of magnitude by positive ion electrospray ionisation-mass spectrometry (ESI-MS) compared to NAs analysed by negative ion ESI-MS. The improved response allowed
multistage ESI-MS (ESI-MSⁿ) experiments to be carried out on unique standards providing
detailed fragmentation pathways for individual NAds.

NAs extracted from crude oil samples and Athabasca tar sands, by solid phase extraction
(SPE), were derivatised to NAds and analysed by high performance liquid
chromatography/ESI-MSⁿ (HPLC/ESI-MSⁿ). Data dependent mass spectrometry provided
unique MS² spectra of individual NAds from which identification of the molecular
formulae, degree of unsaturation (number of rings) and alkanoate chain length of the
amides was possible. The predominant alkanoate chain length was found to be acetamide,
a finding that is compatible with biodegradation studies herein where metabolite end­
points contained an ethanoic acid moiety. This is consistent with beliefs that NAs are
recalcitrant mixtures resulting from the biodegradation of crude oil hydrocarbons. The
level of molecular characterisation achieved in the present study moves significantly
beyond that previously (e.g. molecular weight, formula and comparisons of congener
distributions) and promises to provide valuable structural information for future toxicity,
biodegradation and corrosion studies.

6.2 Suggestions for future work

The major metabolites produced during the biodegradation of iso-, n-, sec- and tert­
BCHBA warrant further investigation. Synthesis of the tentatively identified ethanoic acid
analogs of butanoic acid isomers will primarily allow confirmation of the structures
through NMR and co-injection with the metabolites. A suggested route for the synthesis
might involve the Friedel-Crafts alkylation of phenyl acetic acid. Such reactions are
common and well understood (Vogel, 1989; March, 1985) and a suggested synthetic
strategy is presented below (Figure 6.1). Once synthesised and the structure confirmed,
further toxicity and biodegradation assays would help establish the fate of the more
recalcitrant NAs found in the environment. In particular, isolation of bacteria capable of
degrading this type of more resistant structure holds promise for their environmental
bioremediation.
Further synthesis of environmentally relevant bicyclic and polycyclic surrogate NAs via Friedel-Crafts acylation of appropriate aromatic starting products (using \(\gamma\)-valerolactone in the presence of aluminium chloride catalyst; Section 2.7) would supplement the suite of recalcitrant surrogate NAs to be used in the search for bacteria capable of bioremediation of recalcitrant environmental mixtures.

Although not included in the current work, the bacterial populations were monitored at genetic level during the biodegradation of \(\text{iso-},\ \text{n-},\ \text{sec-}\) and \(\text{tert-}\text{BCHBA}\). Changes in populations were observed coinciding with the degradation. If used with future biodegradation studies of recalcitrant NAs, this technique promises to be a major advance towards finding bacteria capable of degrading such compounds and thus the ultimate bioremediation goal.

Upon hydrogenation of the aromatic intermediates in the synthesis of the BCHBA isomers, two diastereomers were formed. It was not possible to characterise the individual diastereomers by NMR (although this maybe possible with LC-NMR [e.g. Exarchou et al. 2005] which was unavailable in the present study), and thus the subsequent experiments utilised a mixture of the two. The two diastereomers were present in an approximately 2:1 ratio and during the biodegradation experiment, the less abundant isomer degraded proportionally quicker than the more abundant one. An adaptation of the preparative separation used in Section 2.7.1 would allow separation and collection of the individual
diastereomers which in turn would allow spectroscopic characterisation of the individual isomers by NMR to shed light on the difference in degradation rates observed, and possibly be beneficial in predicting levels of biodegradation. It would also be very interesting to perform the toxicity assay on the individual diastereomers to note any differences in toxicity.

Aromatic acids are thought to only comprise a minor amount of NA mixtures. However their toxic effects and biodegradability should still be established. The toxicity and biodegradation assays used herein could be applied to the aromatic synthetic intermediates (e.g. *n*-, *iso*-, *sec*- and *tert*-butylphenylbutanoic acid; Chapter 2) to establish and compare toxicity and biodegradation levels of aromatic NAs to equivalent alicyclic NAs.

The development and application of amide derivatisation to NA characterisation holds much promise for molecular characterisation of individual NAs. The synthesis and derivatisation of more surrogate NAs will be invaluable in building up greater knowledge of amide fragmentation, and so allow more detailed characterisation of individual unknown compounds.

The derivatisation of individual standards was achieved in quantitative yields. However it is not known if the same efficiency was achieved with the complex NA mixtures. The reaction therefore needs to be optimised and monitored for mixtures ultimately allowing the method to be qualitative and quantitative at the same time. Instrument limits of detection for the NAds also needs to be established.

The present study achieved separation on a C18 HPLC column. However the separation could be further developed by investigating the use of different stationary phases, thus potentially achieving separation based on different characteristics.

The LC-ESI-MS method established herein significantly advances NA characterisation beyond previous identification of homologous series, allowing for the first time
characterisation of NAs at a molecular level. The method promises to greatly advance future NA toxicity, biodegradation and corrosion studies.
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Appendix 1

Figure A.1. $^{13}$C NMR spectrum of iso-butylbenzene. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated methanol).

Figure A.2. $^{13}$C DEPT NMR spectrum of iso-butylbenzene. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated methanol).
Figure A.3. $^{13}$C NMR spectrum of $n$-butylbenzene. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform).

Figure A.4. $^{13}$C DEPT NMR spectrum of $n$-butylbenzene. (Joel EX270MHz HR FT-NMR, samples dissolved in deuterated chloroform).
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Date............20/06/06..............