Storyline: answers to assignments

1 **a** $C_2H_5OH + 3O_2 \rightarrow 2CO_2 + 3H_2O$
$\Delta H_c^{\ominus} = -1367\,kJ\,mol^{-1}$

b **i** $C_2H_5OH + O_2 \rightarrow CH_3COOH + H_2O$

ii $\Delta H^{\ominus} = \Delta H_f^{\ominus}(CH_3COOH) + \Delta H_f^{\ominus}(H_2O) - \Delta H_f^{\ominus}(C_2H_5OH)$
$\Delta H^{\ominus} = (-485\,kJ\,mol^{-1}) + (-286\,kJ\,mol^{-1}) - (-277\,kJ\,mol^{-1})$
$= -494\,kJ\,mol^{-1}$

c The products are most likely to be a mixture of water, ethanoic acid and carbon dioxide.

d Ethanol can also be exhaled or excreted.

2 **a** $K_c = \dfrac{[C_2H_5OH(g)]}{[C_2H_5OH(blood)]}$

b The values will be those corresponding to the inside of the lungs,
ie pressure = 1 atm, temperature = 37.0 °C.

c Since the concentrations are measured in the same units in the aqueous and gaseous phases, the gaseous ethanol concentration in equilibrium with 80 mg per 100 cm^3 of blood corresponds to
$(4.35 \times 10^{-4} \times 80)$ mg per 100 cm^3 of air =
3.48×10^{-2} mg per 100 cm^3 of air.

3 **a** $O_2(g) + 4H^+(aq) + 4e^- \rightarrow 2H_2O(l)$: reduction
$C_2H_5OH(aq) + H_2O(l) \rightarrow CH_3COOH(aq) + 4H^+(aq) + 4e^-$: oxidation

b Reduction occurs at the positive electrode.
Oxidation occurs at the negative electrode.

4 **a** The absorption bands of water vapour are likely to be broad and so obscure most of the spectrum above 3000 cm^{-1}. The ethanol band which occurs just below 3000 cm^{-1} is clear of the water absorptions and is thus suitable for ethanol detection.

b The common bands arise from vibrations of the OH group which is common to both molecules.

c The C–H bond.

d Propanone also contains C–H bonds and so will also absorb close to 3000 cm^{-1}.

5 **a** $H_3\overset{+}{N}-CH_2-CH_2-CH_2-COO^-$

b There is no chiral centre in GABA because the $-NH_2$ and –COOH groups are attached to different carbon atoms.

6 **a** Phenol group
Secondary alcohol group
Secondary amine group

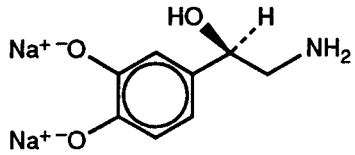
b The only difference is in the amine group. Noradrenaline has a primary amine group. Adrenaline has an additional methyl group making the amine group secondary.

c **i** Isoprenaline contains a secondary amine group in place of the primary amine of noradrenaline.

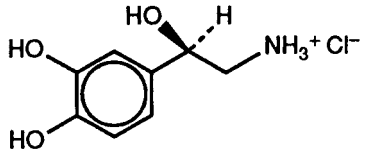
ii Salbutamol contains a secondary amine group and a primary alcohol group in place of one of the phenol groups.

d **i** The presence of the phenol groups would produce a strong coloration (probably purple) with $FeCl_3(aq)$.

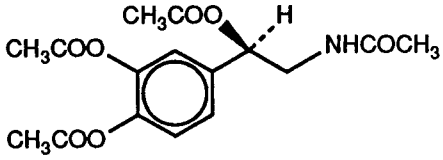
ii The weakly acidic phenol groups would protonate the hydroxide ions to produce water and



iii The primary amine group will be protonated by the acid to produce

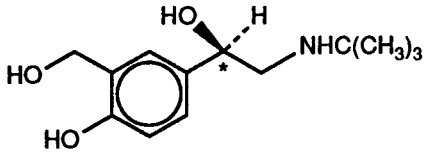


iv The amine *and* alcohol and phenol groups will react. The products will be HCl, H_2O and



e Salbutamol contains phenol, secondary alcohol and secondary amine groups. These groups will all react in a similar way to the noradrenaline groups.

7 **a** The group of atoms around the carbon atom marked with an asterisk confers chirality.



b The receptor site is also chiral. It is likely that one isomer fits better than the other and places the important functional groups in a better position to interact.

8 **a** The side-chain is longer in propranolol with the inclusion of the $-OCH_2-$ group.
The phenolic and $-CH_2OH$ groups on the benzene ring in salbutamol are replaced by a second aromatic ring in propranolol, changing the size and electron distribution of this part of the molecule.

b **i** No effect. The pharmacophore is absent.

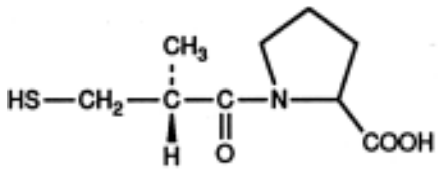
ii Probably an agonist. An arrangement very similar to the pharmacophore is present.

iii Antagonist. The pharmacophore is modified in a way similar to propranolol.

9 Asp Arg Val Tyr Ile His Pro Phe and His Leu

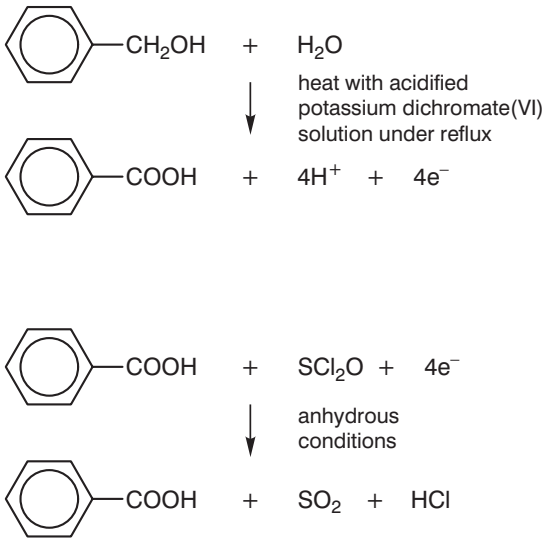
10 **a** The carbon atom to which the methyl group is attached forms a chiral centre in the molecule. There is an optical isomer of captopril in which the $-CH_3$ group points in the opposite direction (behind the plane of the paper as the molecule is represented in the **Storyline**). This isomer does not interact as favourably with the ACE enzyme.

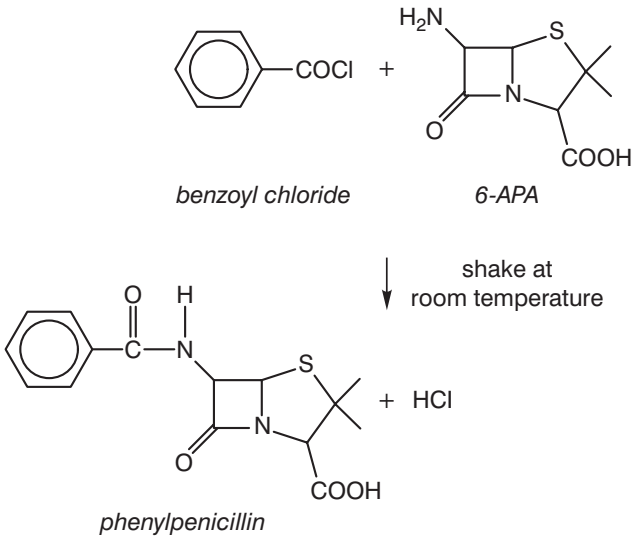
b



c The –COOH group.

11





(In practice, a solution of the *sodium salt* of 6-APA is shaken with benzoyl chloride. The pH of the mixture is kept in the range pH 5–8.)

12 **a** Mutations in a bacterial population naturally produce strains which are resistant to penicillin. These strains survive treatment with penicillin, and so develop at the expense of strains which are destroyed by penicillin.

b The infection must be eradicated as far as possible, to prevent small numbers of mutants multiplying in an environment that gives them an advantage.

13 **a** Common features: fused 4- and 5-membered rings
β-lactam ring
–COOH in corresponding position
O and S atoms (both Group 6 elements) in equivalent positions
same stereochemistry and ring strain.

b Clavulanic acid is taken up preferentially by the β-lactamase enzyme and blocks the active site, so that the penicillin molecule is not attacked.
(But, note that the differences between the clavulanic acid molecule and a penicillin molecule are such that clavulanic acid is not recognised by the active site on the cross-linking enzyme in the bacterial cell walls.)

Activities: notes and answers to questions

MD1.1 Aldehydes and ketones

Safty note Information about hazardous chemicals is given on the activity sheet.

Advance preparation

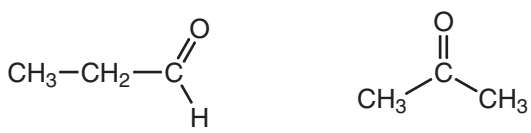
The melting point of 2-methylpropan-2-ol (*t*-butyl alcohol) is 25.5 °C. If kept in a cold store, it will take some time to melt.

Note This experiment is similar to **Activity WM3 Part 2**. We suggest that **MD1.1** is included if **WM3** has not been done or if you feel that it will help revise the reactions of aldehyde and ketones at this stage of the course.

a Propan-1-ol, propan-2-ol

b Dichromate(VI) ions (orange) are reduced to chromium(III) ions (green) by the alcohols.

c

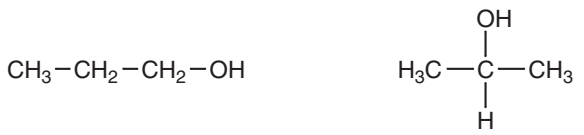


d

e **i** oxidation to carboxylic acid.

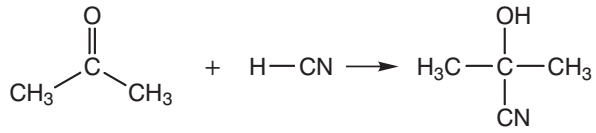
ii Similar process to **d** above, but Cu^{2+}(aq) ions reduced to Cu(I) oxide in place of the reduction in **b**.

f



propan-1-ol *propan-2-ol*

g



MD1.2 BAC determination using gas–liquid chromatography

a

alcohol	formula	retention time/min
methanol	CH_3OH	1.2
ethanol	CH_3CH_2OH	1.4
propan-1-ol	$CH_3CH_2CH_2OH$	2.3
butan-1-ol	$CH_3CH_2CH_2CH_2OH$	3.7
pentan-1-ol	$CH_3CH_2CH_2CH_2CH_2OH$	5.5

b The retention time increases for each additional CH_2 in the chain. Hexan-1-ol would therefore be expected to have a retention time of about 7.3 minutes.

Note

The time for methanol is anomalous as the stationary phase was polar and the C_1, alcohol proportionally has stronger H bonding with it.

c Ratio of ethanol : propan-1-ol = 1 : 3.5 in trace I

d Ratio of ethanol : propan-1-ol = 1 : 7.0 in trace II
= 1 : 2.0 in trace III

These are equivalent to BACs of 40 in trace II
140 in trace III

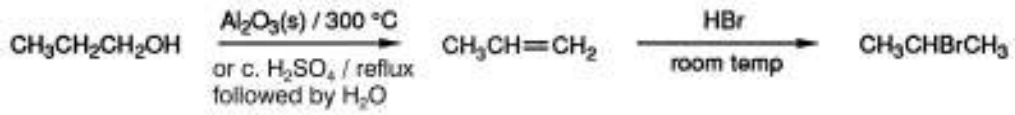
Trace III would correspond to a suspect who was 'over the limit'.

e The determination is likely to become less reliable as the time interval between the sample and reference peaks increases owing to possible fluctuations in conditions. Of the two peaks adjacent to ethanol, the methanol peak is rather close and can interfere with the ethanol peak causing inaccuracies. Propan-1-ol is therefore chosen.

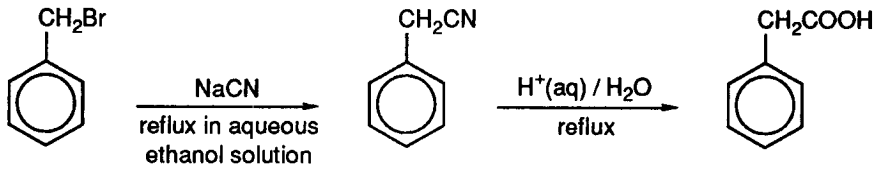
MD3.1 Making a toolkit of organic reactions

The completed flow sheets of the toolkit are shown on pages 111 and 112.

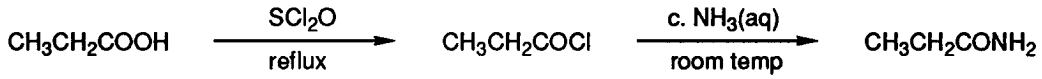
a i



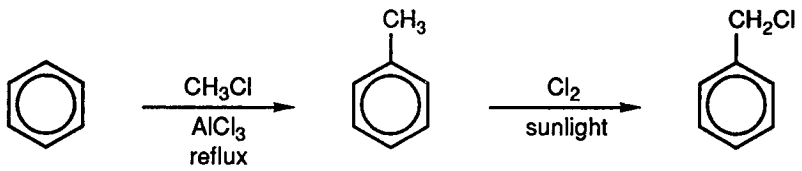
ii



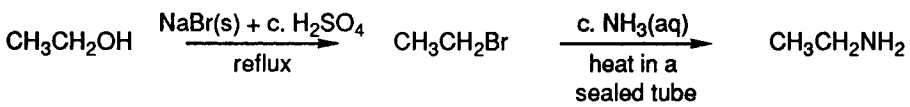
iii



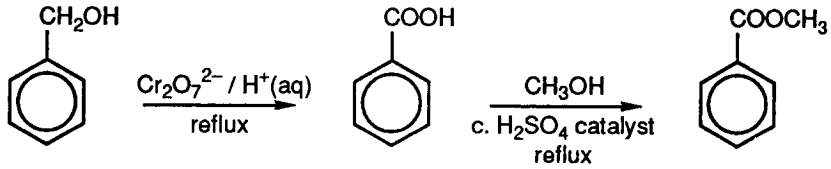
iv



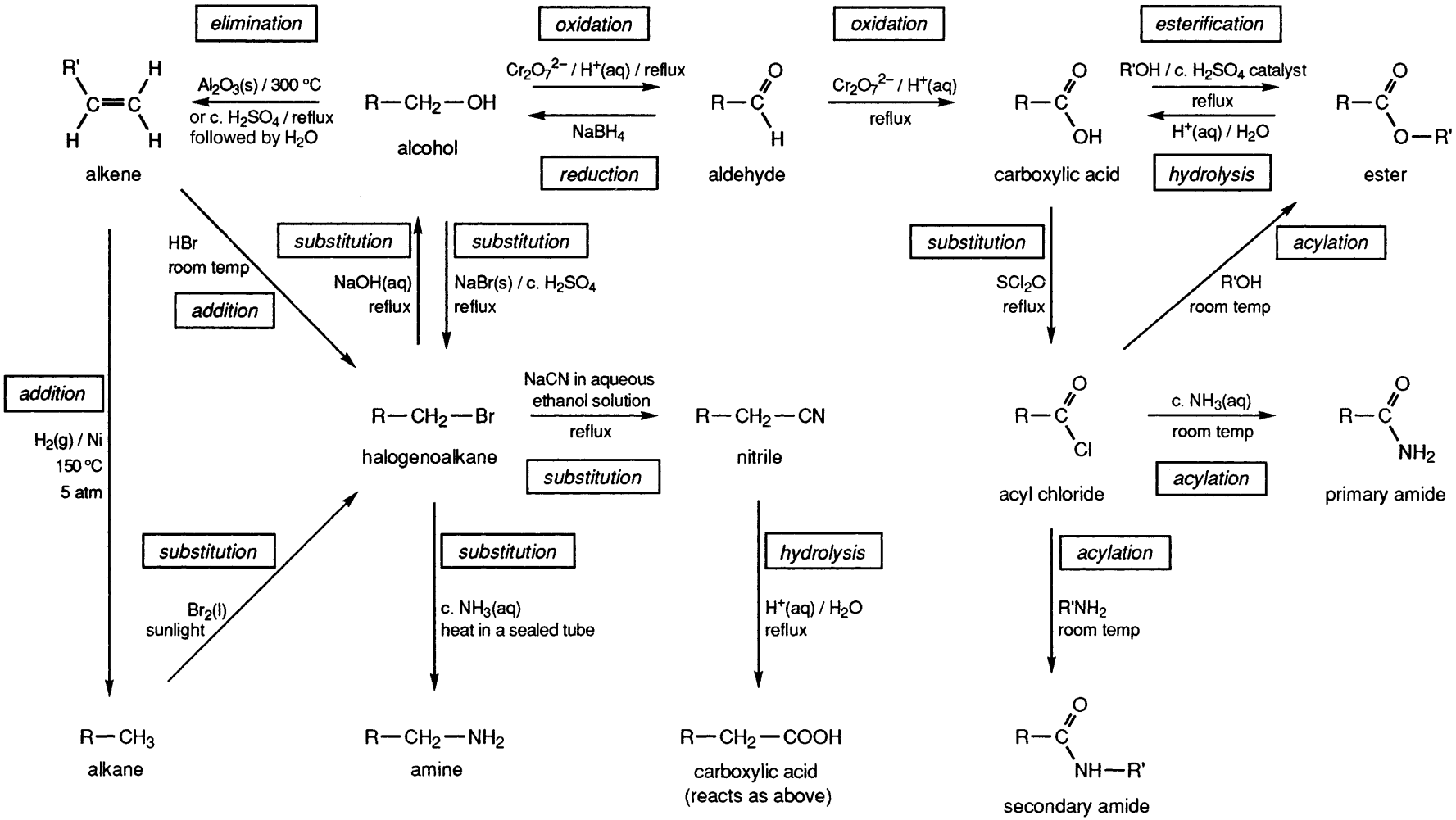
v



b i



The toolkit: Chart A



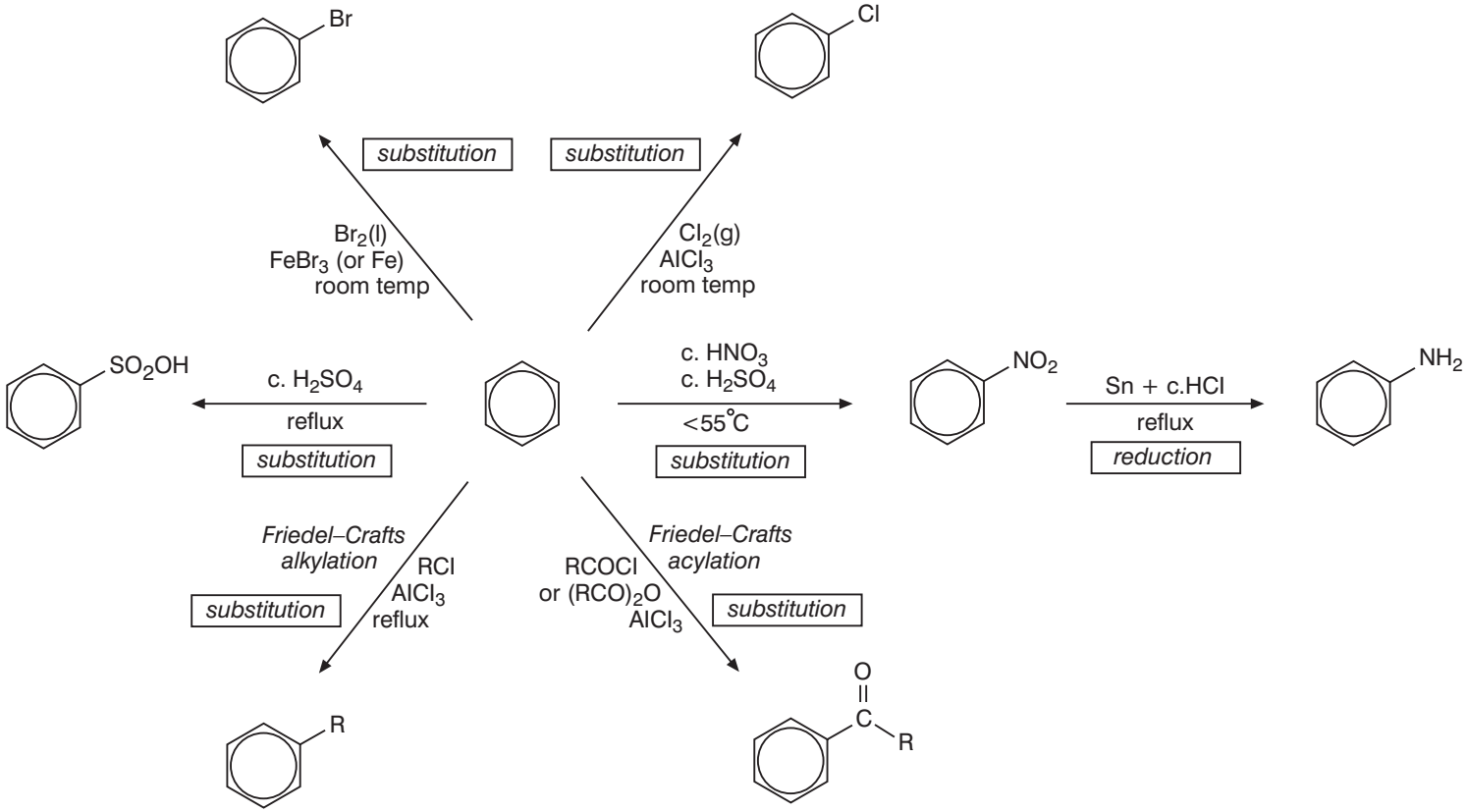
Notes

1. The formation of a nitrile from a halogenoalkane is a *carbon–carbon bond forming reaction*. The carboxylic acid formed from the nitrile has an extra carbon atom in the side-chain. All the other reactions are simple *functional group interconversions*.

2. The halogenoalkane shown will only be a minor product of the reaction from the alkene. The main product will be the isomer with the Br atom attached to the second carbon atom.

3. Students may wish to add other reactions to this toolkit, for example the formation of a secondary alcohol from an alkene RCH═CHR' and thence oxidation to a ketone.

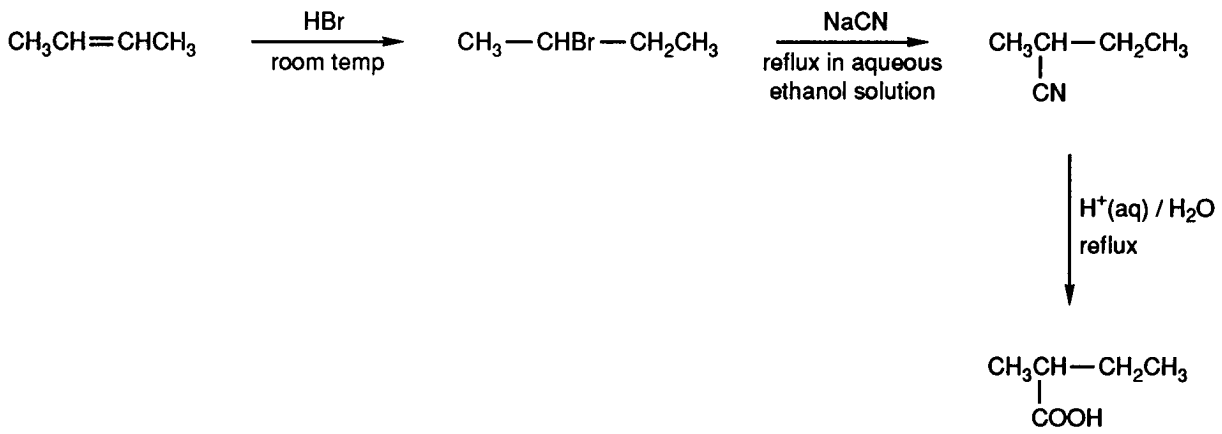
The toolkit: Chart B



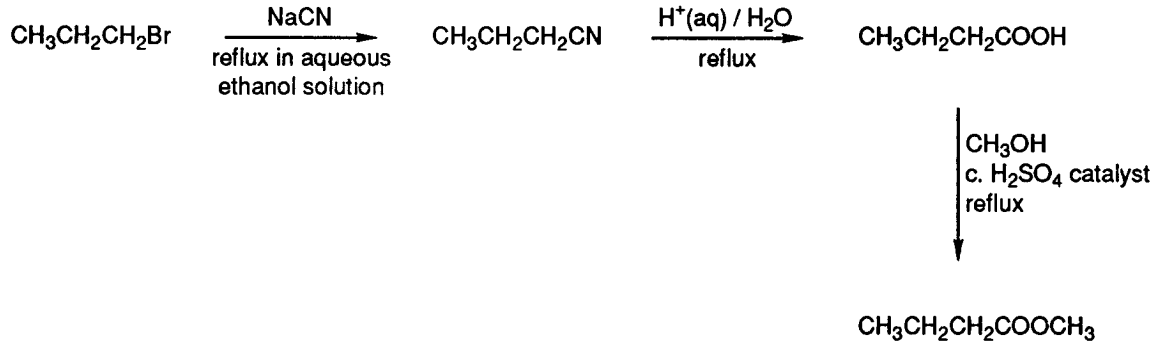
Notes

1. The Friedel–Crafts reactions are *carbon–carbon bond forming reactions*.
2. All the substitution reactions of arenes are *electrophilic*.

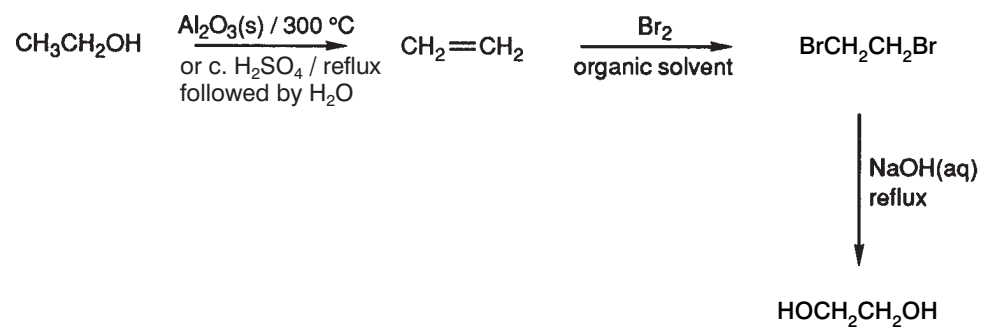
ii


CH3CH=CHCH3
HBr
room temp
CH3—CHBr—CH2CH3
NaCN
reflux in aqueous ethanol solution
CH3CH—CH2CH3
CN
H+(aq) / H2O
reflux
CH3CH—CH2CH3
COOH

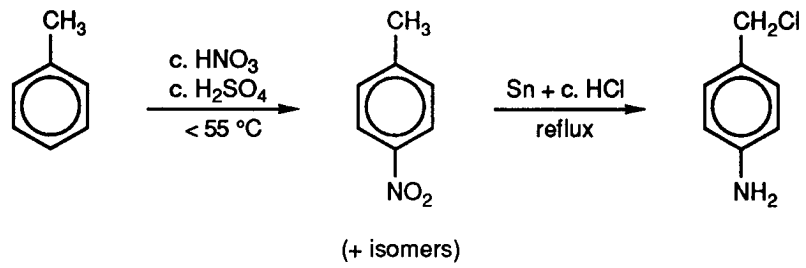
iii


CH3CH2CH2Br
NaCN
reflux in aqueous ethanol solution
CH3CH2CH2CN
H+(aq) / H2O
reflux
CH3CH2CH2COOH
CH3OH
c. H2SO4 catalyst
reflux
CH3CH2CH2COOCH3

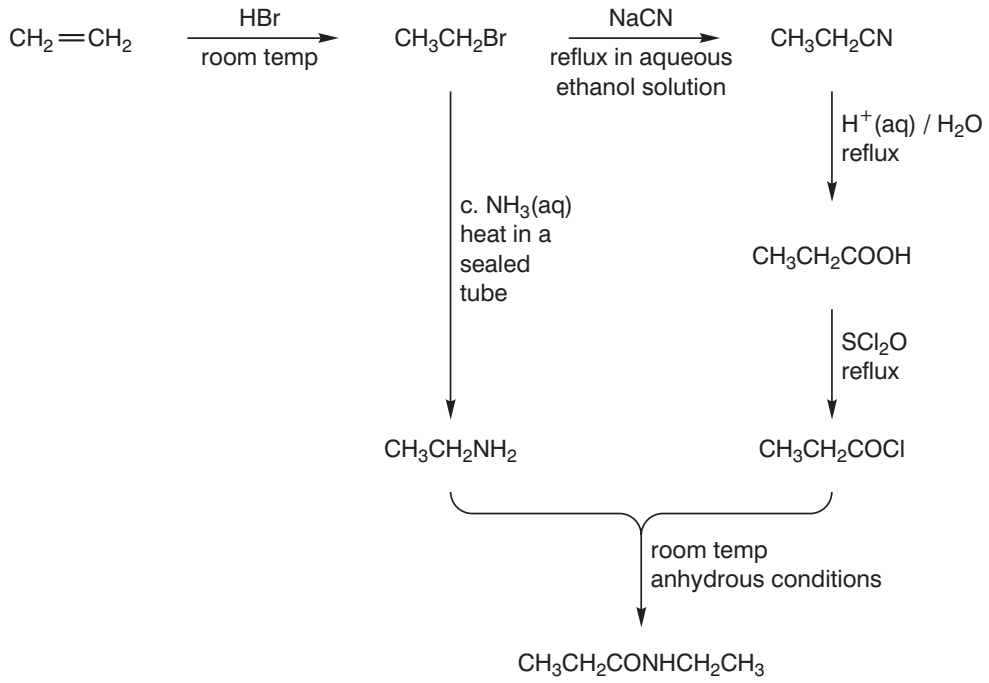
iv


CH3CH2OH
Al2O3(s) / 300 °C
or c. H2SO4 / reflux
followed by H2O
CH2=CH2
Br2
organic solvent
BrCH2CH2Br
NaOH(aq)
reflux
HOCH2CH2OH

v


CH3
c. HNO3
c. H2SO4
< 55 °C
CH3
NO2
(+ isomers)
Sn + c. HCl
reflux
CH2Cl
NH2

c



MD3.2 Classifying reactions

This activity is based on the toolkits produced from **MD3.1** and will enable students to familiarise themselves with them.

Below is an example of a completed table.

Type of reaction		Name of homologous series	Functional group	Example of reaction
Hydrolysis				
	i	Ester	$-C(=O)-O-R$	$CH_3COOC_2H_5 + H_2O \xrightarrow{H^+} CH_3COOH + C_2H_5OH$
	ii	Amide	$-C(=O)-NH_2$	$CH_3CONH_2 + H_2O \xrightarrow{OH^-} CH_3COOH + NH_3$
	iii	Nitrile	–CN	$CH_3CN + 2H_2O \xrightarrow{H^+} CH_3COOH + NH_3$
Esterification	**i**	Carboxylic acid	–COOH	$RCOOH + R'OH \xrightarrow{H^+} RCOOR' + H_2O$
Elimination	**i**	Primary and secondary alcohols	$-CH_2OH$ and $>CHOH$	$CH_3CH_2OH \rightarrow C_2H_4 + H_2O$
Acylation	**i**	Alcohol	–OH	$C_2H_5OH + CH_3COCl \rightarrow CH_3COOC_2H_5 + HCl$
	ii	Amine	$-NH_2$	$CH_3NH_2 + CH_3COCl \rightarrow CH_3CONHCH_3 + HCl$
	iii	Ammonia	$-NH_3$	$NH_3 + CH_3COCl \rightarrow CH_3CONH_2 + HCl$
	iv	Arene	–	$C_6H_6 + CH_3COCl \rightarrow C_6H_5COCH_3 + HCl$
Addition				
electrophilic	**i**	Alkene	$>C=C<$	$C_2H_4 + Br_2 \rightarrow BrCH_2CH_2Br$
nucleophilic	**ii**	Aldehyde and ketone	–CHO and $>C=O$	$CH_3CHO + HCN \rightarrow CH_3CH(OH)CN$
Substitution				
radical	**i**	Alkane	–	$CH_4 + Cl_2 \rightarrow CH_3Cl + HCl$
electrophilic	**ii**	Arene	–	$C_6H_6 + Br_2 \rightarrow C_6H_5Br + HBr$
nucleophilic	**iii**	Halogenoalkane	–X (Cl, Br, I)	$C_2H_5Br + OH^- \rightarrow C_2H_5OH + Br^-$
Oxidation	**i**	Primary alcohol	$-CH_2OH$	$CH_3CH_2OH \rightarrow CH_3CHO + 2H^+ + 2e^-$
	ii	Aldehyde	–CHO	$CH_3CHO + H_2O \rightarrow CH_3COOH + 2H^+ + 2e^-$
Reduction	**i**	Aldehyde	–CHO	$CH_3CHO + H_2 \rightarrow CH_3CH_2OH$
	ii	Alkene	$>C=C<$	$C_2H_4 + H_2 \rightarrow C_2H_6$

a Alcohol, carboxylic acid

b Amine, amide

c Alcohols, carboxylic acids

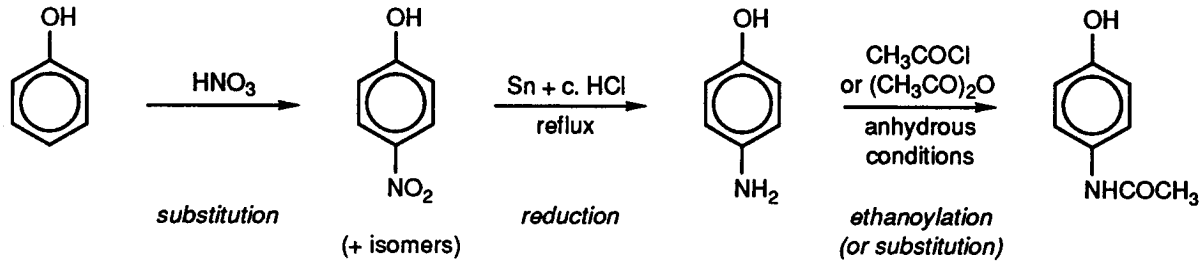
d $H_2(g)$; $CH_3CN + 2H_2 \rightarrow CH_3CH_2NH_2$ (nickel as catalyst)
$NaBH_4$; $CH_3CHO \rightarrow CH_3CH_2OH$

e To add a C–C bond
For example:

$$\underset{\textit{iodo}\textbf{\textit{methane}}}{CH_3I} \xrightarrow{KCN} \underset{\textit{ethanenitrile}}{CH_3CN} \xrightarrow{H_2} \underset{\textit{amino}\textbf{\textit{ethane}}}{CH_3CH_2NH_2}$$

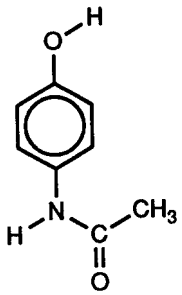
MD3.3 Using the toolkit to synthesise medicines

Part 1: Making paracetamol from phenol



Students will probably use a mixture of concentrated nitric acid and concentrated sulphuric acid to nitrate phenol, so assume this is reasonable.

a

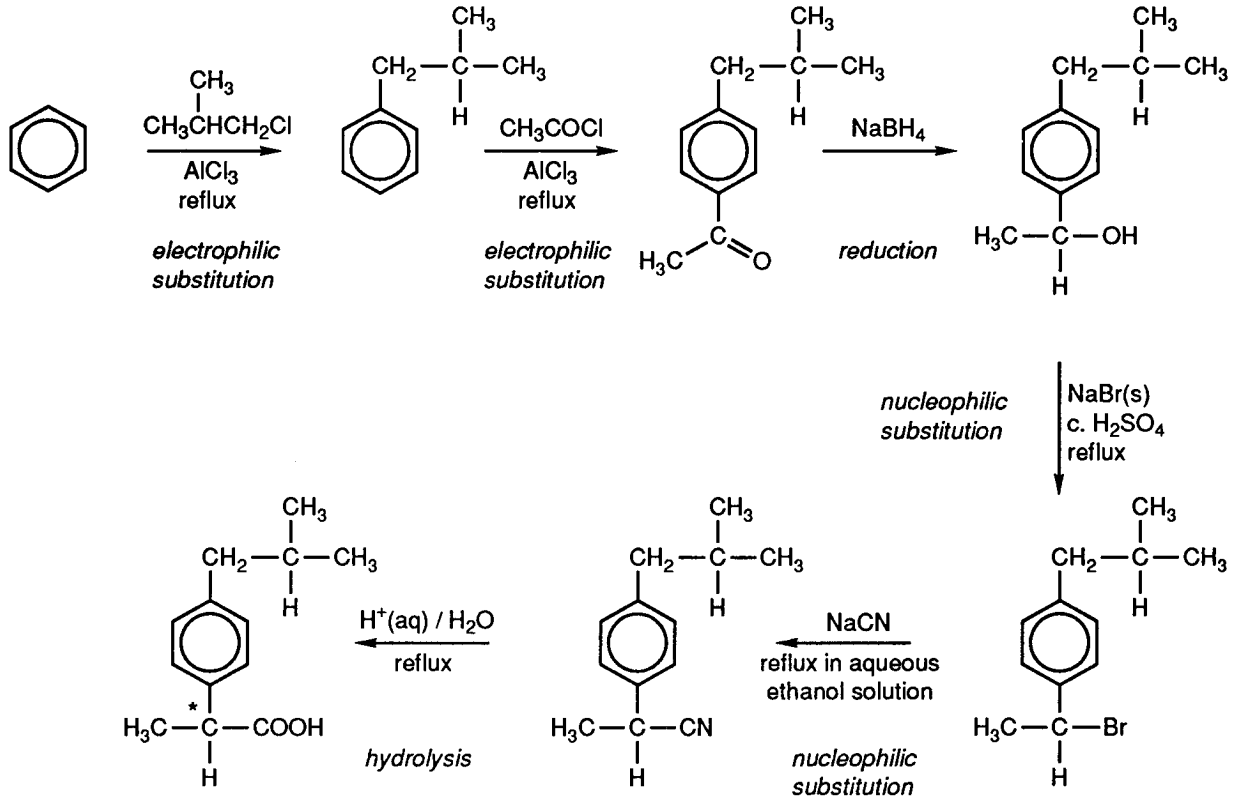


b See above.

c Recrystallise from water. (Dissolve the crystals in the minimum amount of hot water. Filter to remove any solid impurities. Allow to cool and crystallise. Filter. Wash crystals with cold water.)

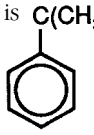
d The pure compound should melt sharply at 169 °C, or you could use thin-layer chromatography to compare crystals with a pure sample of paracetamol.

Part 2: Making ibuprofen from benzene

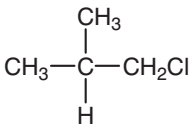


Note

This is not the route used to make ibuprofen commercially. The first step in the above scheme gives low yields of the desired product. A rearrangement take place and the main product is $C(CH_3)_3$.



e

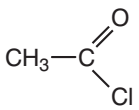


1-chloro-2-methylpropane

f Electrophilic substitution reaction

g Anhydrous $AlCl_3$ helps to polarise the halogenoalkane

h



ethanoyl chloride

i Anhydrous $AlCl_3$

j Reaction with CN^- ions

k The key intermediate before CN^- substitution can take place is the corresponding halogenoalkane.

l The ketone must be reduced to an alcohol before the halogenoalkane can be prepared.

m and **n** See above

Part 3: Analysing spectra

o **i** Below 3000 cm^{-1}, C–H bonds in the alkyl groups; above, in aryl groups.

ii At 0.9, two identical C**H**$_3$ groups.
At 1.9, one C**H** alkyl group.
At 2.5, one C**H**$_2$ group.
At 7.1–7.3, five C**H** hydrogens in an aryl ring, thus a monosubstituted compound.

iii 134, molecular ion peak
91, loss of 43, $(CH_3)_2CH$
There are 9 C atoms, each contributing *ca* 1.1% to (M+1) peak

p **i.r.** The significant change is at *ca* 1700 cm^{-1} (carbonyl) introduced into the spectrum of **B**.

n.m.r. There are two changes. There is an extra methyl group (at 2.6 probably adjacent to CO) and there are two sets of signals at 7.2 and 7.8 indicating that there are two sets of two H atoms in the aryl ring with different environments, suggesting a 1,4-disubstituted arene.

m.s. The mass spectrum of **B** also shows a mass peak (176), the loss of 15 (CH_3) to give a large peak at 161. The peaks at 134, 91 and 43, similar to those in **A** indicate that the **A** has another substituent on the aryl ring, in the 4-position and that it is CH_3CO.

q **i** **i.r.** The tell-tale strong absorption in **B** at *ca* 1700 cm^{-1} (carbonyl) is missing. Instead there is strong to broad absorption at *ca* 3500 cm^{-1} indicating an –OH (alcohol).

n.m.r. Key differences in the spectrum of **C** include the extra broad absorption at 2.0 (O**H**) and absorption at 4.9, which is indicative of a –C(–)– **H** group.
The signal due to the methyl group at 2.6 in **B** is not present in the spectrum of **C**, but a new methyl signal has appeared at 1.4 (adjacent to the alcohol group).

m.s. The mass peak of **C** is 178, two more than that for **B**. The peak at 163 corresponds to that of 161 for **B**. The mass spectrum of **C** has several

peaks which are the same as **B**, eg at 91 and 43, indicating a similar structure.

ii Overall it appears that a C=O group in **B** has been **reduced** to CHOH in **C**.

r **n.m.r.** The signals at 0.9 (6**H**), 1.8 (1**H**) and 2.4 (2**H**) are still present indicating the grouping

$$-CH_2-CH(CH_3)_2$$

The signals for the aryl hydrogen atoms at 7.1 and 7.2 are still the same. The signals at 1.5 (3**H**) and 3.8 (1**H**) indicate

$$CH_3-\underset{|}{C}H-$$

i.r. Absorbance at *ca* 1700 cm^{-1} has reappeared (carbonyl) and that at *ca* 2900 cm^{-1} indicates the carboxylic acid (–OH) (although there is no signal in n.m.r. for this hydrogen atom).

m.s. Molecular ion peak at 206 with major peaks at 163 and 161 indicating loss of 43 [$(CH_3)_2CH$] and 45 (COOH). The peak at 119 is probably loss of CO_2 from 163.

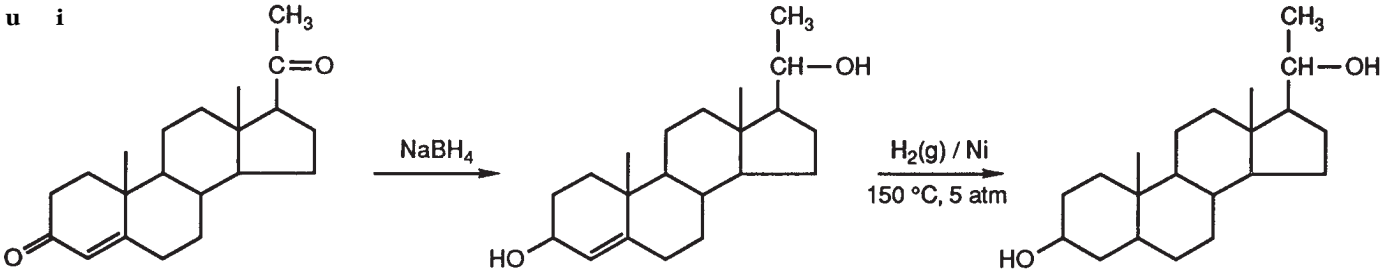
(This is not an easy spectrum to analyse and answers showing understanding of the peaks at 206, 163 and 161 should be considered good.)

Part 4: Investigating sex hormones

s Secondary alcohol

t Oestradiol contains a phenolic –OH group and will give a purple complex when shaken with a neutral solution of Fe^{3+} ions. Testosterone will give no marked coloration with $FeCl_3$ solution.

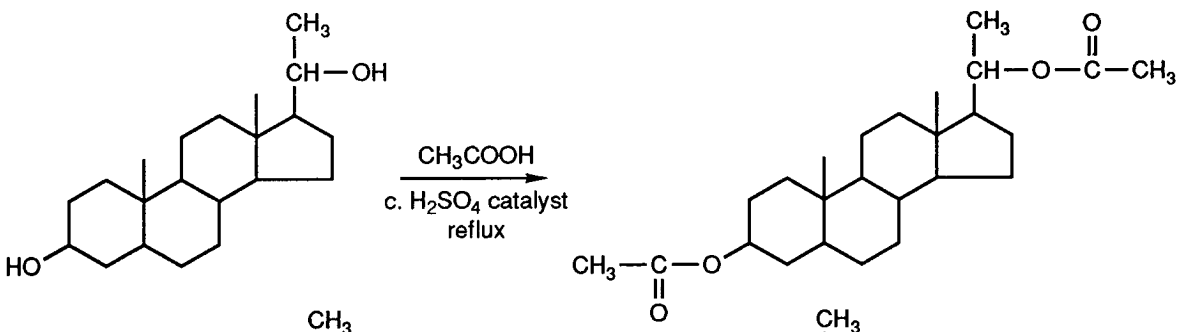
u **i**



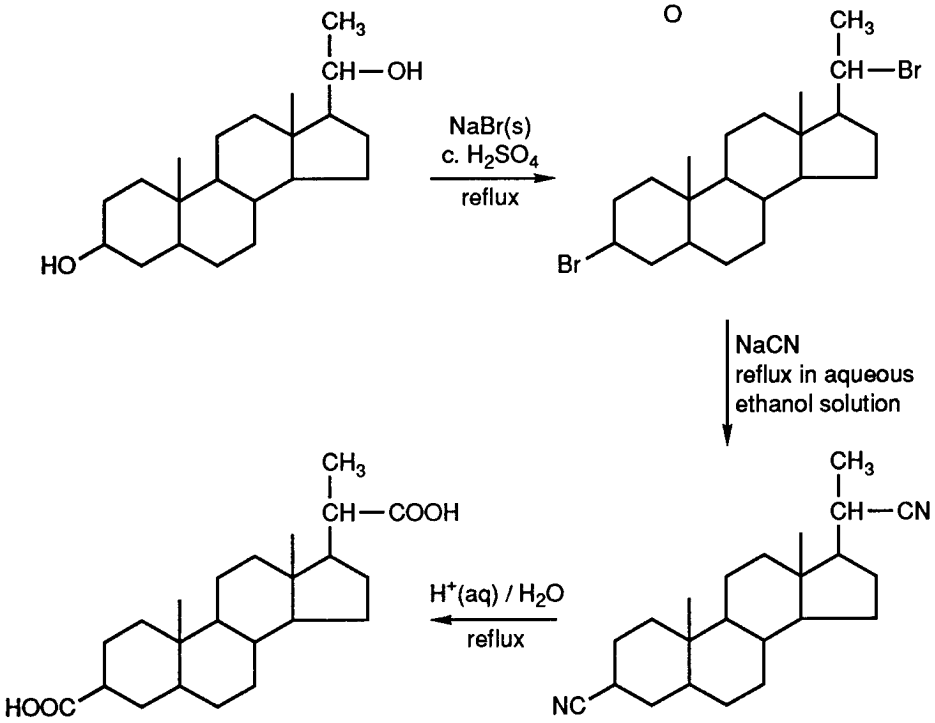
Note

In fact, both reductions can be accomplished together, using hydrogen with a nickel catalyst, but students probably will not know this.

ii



iii



v The steroid is probably progesterone.
Strong peaks at 1700 cm^{-1} and 1680 cm^{-1} due to the presence of two different C=O groups. No –OH absorption around 3500 cm^{-1}.

MD3.4 Manufacturing salbutamol (optional extension)

Part 1: Costing salbutamol

Compound	M_r	Yield/%	Mass produced/g	Amount produced/ moles
aspirin	180	–	1000	5.56
A	194	85	*916*	*4.72*
B	194	60	*550*	*2.83*
C	273	75	*580*	*2.13*
D	265	55	*310*	*1.17*
salbutamol	239	60	*168* (mixture isomers)	*0.70*
		30	*84* (salbutamol)	*0.35*

MD3.4 Table 1 Yields and quantities for salbutamol synthesis

Reagent/solvent	Quantity required	Cost/£
aspirin	1 kg	*12.80*
methanol	*10 dm^3*	*42.00*
sulphuric acid	*0.01 kg*	*0.02*
nitrobenzene	*9.16 dm^3*	*93.00*
aluminium chloride	*0.63 kg*	*7.06*
trichloromethane	*5.5 dm^3*	*58.85*
bromine	*0.45 kg*	*5.94*
2-amino-2-methylpropane	*0.31 kg*	*2.98*
ethoxyethane	*12.4 dm^3*	*74.40*
lithium tetrahydridoaluminate	*0.13*	*58.50*
	Total cost	*355.55*

MD 3.4 Table 2

Cost of materials to synthesise 1 kg of salbutamol = £4244

a There would be many other costs, eg energy, equipment, salaries, formulation, containers, transport. In addition, there would be the cost of separating salbutamol from its optical isomer.

b 77%. The solvents could be distilled off and recycled.

Part 2: Spectra of compounds involved in the synthesis of salbutamol

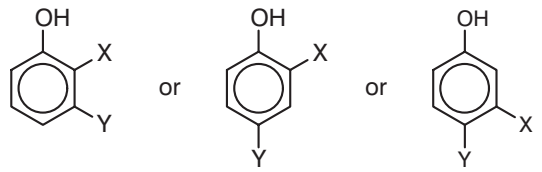
c **i** The sharp peaks at 1690 cm^{-1} and 1750 cm^{-1} indicate the presence of carbonyl groups. (The peak at 1690 cm^{-1} is due to the carbonyl in the carboxylic acid group; the peak at 1750 cm^{-1} is due to the carbonyl in the ester group.)
The broad peak at *ca* 3000 cm^{-1} is typical of an –OH group in a phenol and/or carboxylic acid.

ii At 2.2: the C**H**$_3$ group.
At 13.1: the –COO**H** group

iii At 180 – molecular ion peak
At 163 – loss of OH (–17)
At 120 – loss of $C_2H_4O_2$ (actually loss of CH_2CO followed by H_2O) from molecular ion
At 43 – the appearance of CH_3CO^+ ion

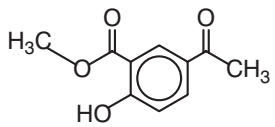
d The key feature of the i.r. spectrum is the change at *ca* 3000 cm^{-1} indicating the loss of a phenolic hydroxyl and/or carboxylic acid group.
The n.m.r. spectra show the loss of a carboxylic acid –O**H** group, and the gain of a methyl group. The C**H**$_3$ group is in an environment different to that in aspirin. It is bonded to an oxygen atom (see data sheet).
The mass spectrum shows the molecular ion peak has shifted by 14 (H replaced by CH_3). This ion loses 31 (CH_3O rather than OH) and 42 (CH_2CO). The 152 ion loses 32 (CH_3OH rather than 18, H_2O, from 138 in aspirin).

e **i** They are isomers.
ii The major difference in the i.r. is the reappearance of a broad peak at *ca* 3200 cm^{-1} indicating that there is once again a hydroxyl group, either as a phenol or as a carboxylic acid.
The n.m.r. suggests a phenolic –O**H** at 11.1, two methyl groups in different environments (at 2.4 and 3.9) and three different environments for only 3 H atoms on the aryl ring.
Note the shift for the CH_3 absorption from 2.2 to 2.5 (as it changes its environment).
Thus, the structure so far appears to be



Note

Working back from the structure of compound **C**, the structure of compound **B** is probably



f **i** One of the C**H**$_3$ protons in B has been replaced in forming **C**. (The absorption is further downfield indicating the replacement of the H atom by a more electronegative atom (Br).)

ii Natural bromine contains two isotopes of equal abundance, 81 and 79.

g Students could identify the absorptions at 6.6, 7.0 and 7.3 as C–**H** arene ring, there being three different environments for them.
The peak at 1.0 is due to –C(C**H**$_3$)$_3$, that at 2.5 to –C**H**$_2$– adjacent to the nitrogen and that at 4.5 to C**H**$_2$– adjacent to the –O**H** group.
(The other absorptions at 9.0, 4.9, 4.4 and 3.3 are due to the various –O**H** groups and the –N**H** group being much more difficult to assign as there will be exchanges occurring.)
The spectrum was not 'doctored'. There are impurities at 2.4 and also at 3.3; no integration therefore shown at these two chemical shifts.

MD5.1 Making and testing a penicillin

Safety note Information about hazardous chemicals is given on the activity sheet. The 6-APA is a fine powder and can cause allergic reactions if inhaled. You may prefer to make up the solution of 6–APA for the students prior to the practical. It is best to use a conventional fume cupboard with a good exhaust system (not the recirculating type).

General COSHH regulations cover work with microorganisms as well as the use of chemicals. You should consult your biology department about handling bacterial cultures and preparing agar plates. It is vital that you follow the correct procedure. **Students should be made aware of the rules for handling microbiological material and the reasons for the precautions they are taking.**

Full details of sterile technique are given in:

- *CLEAPSS Laboratory Handbook.* School Science Service, Microbiology Sections 15.2 and 15.12
- *Topics in Safety* 2nd edition, Microbiology p. 26, Association for Science Education (fully revised 1988) (new edition in preparation)
- *Microbiology: an HMI Guide for Schools and Further Education*. DES (1985; reprinted with amendments 1990)

Basic rules to observe when handling bacterial cultures

1. Wash down the work area with a suitable disinfectant before and after the session.
2. Wear gloves and a laboratory coat at all times.
3. Wash your hands well in hot soapy water before and after the session. Make sure beforehand that skin cuts are protected with effective waterproof dressings. Do not touch your face or other parts of your body during a session.
4. Do not open containers *any wider* and do not keep them open *any longer* than is absolutely necessary.
5. Transfer microorganisms as quickly as possible.
6. Do not breathe or cough over a Petri dish when the lid is raised.
7. Plates should be sealed with small pieces of adhesive tape along a diameter (see Figure 2 on page 333 activity sheets), not around the circumference. The plates should remain sealed during the subsequent examination.
8. All cultures, plates, syringes and contaminated apparatus should be sterilised before disposal or before being returned to stock cupboards.

Storing 6-APA

The solid can be stored for many months in a refrigerator, but it will decompose slowly at room temperature. It is best obtained fresh each year. Unused material obtained from SmithKline Beecham should be returned.

Timing

The preparation of the broth culture of *Bacillus subtilis* takes 24 hours, so this must be started the day before the practical session. The agar plates should be poured immediately before the practical session if possible.

The practical part of the activity should be done in one session. The 4-membered lactam ring is easily broken open by strong acids and alkalis, so it is important to control the pH carefully during the synthesis and purification procedures, and to work as quickly and efficiently as possible.

Making up the agar plates

Requirements

Bacillus subtilis living culture on agar
nutrient broth tablets
screw cap bottles (30 cm^3)
inoculating loop
access to incubator
disposable sterile syringes
access to pressure cooker or autoclave
autoclavable plastic bags (or roasting bags)
*sterile Petri dishes with lids (4)
sterilised nutrient agar (80 cm^3)

*If glass Petri dishes are used, these must be sterilised first in a pressure cooker or autoclave. Sterile plastic plates are more convenient, but these are not recyclable.

1. First make a sub-culture of *Bacillus subtilis* in a sterile nutrient broth as follows. Put two nutrient broth tablets and 15 cm^3 distilled water into a 30 cm^3 screw cap bottle. Put the cap on loosely and sterilise in a pressure cooker or autoclave for 30 minutes. Allow to cool.

   Sterilise an inoculating loop in a Bunsen flame until red hot and allow to cool. Draw the sterile loop across the surface of the *Bacillus subtilis* culture on the agar slope, so that a very small amount of the culture is taken up on the loop. Transfer to the sterile nutrient broth. Flame the neck of the bottle to resterilise, by passing it briefly through a flame. Replace the cap and stand the bottle in an incubator set at 25–28 °C for 24 h. Sterilise the loop in a flame. Shake the tube from time to time during the incubation period.

Note

The neck of the culture bottle must be flamed if the cap is removed at any time. The cap should not be placed on the bench. Flaming should be repeated before the cap is replaced.

2. Using a sterile syringe, transfer 2 cm^3 of the broth culture into 20 cm^3 sterilised distilled water in a screw cap bottle. (Each group needs 4 cm^3 of the diluted *Bacillus subtilis* broth culture.)

3. Make up the nutrient agar solution according to the directions given, and sterilise in a pressure cooker or autoclave for 30 minutes. You can do this in bulk or in a number of small bottles. (Agar only dissolves in water above 95 °C. It is best to let the agar soak in distilled water first for about 15 minutes and then heat on a water bath. *Do not add solid agar to boiling water*.)

   Allow the agar solution to cool to about 48 °C before pouring. If the solution is too hot, it will kill the bacteria. If it cools below 40 °C, it will solidify. It is a good idea to have a water bath at about 45 °C to keep bottles of agar solution at the right temperature for pouring.

4. Using a sterile syringe, place 1 cm^3 of the diluted *Bacillus subtilis* broth culture into each sterile Petri dish on a level surface. Add 20 cm^3 sterilised nutrient agar solution to each and mix by swirling gently. (Alternatively, you may find it more convenient to mix the *Bacillus subtilis* broth culture with the nutrient agar in bottles before pouring.) Cover the plates and leave to set. Put the containers from the agar immediately into a bowl of hot water.

Note

The *Bacillus subtilis* is incorporated in the agar in this way to give a uniform distribution of bacteria. This makes it much easier to see any inhibition.

An alternative procedure for testing antibacterial activity

You may find it more convenient to use filter paper discs instead of the cork borer/well method.

You can replace steps **9** and **10** in the activity (pages 332—333) with the following procedure.

Use a hole puncher to make filter paper discs. Place one of the discs in the diluted phenylpenicillin solution. Flame a pair of forceps by holding them in a Bunsen flame until the tips glow red hot. Allow them to cool and then pick up the filter paper disc. Allow the excess solution to drain away. Raise the lid of a Petri dish and place the disc on the surface of the agar. Replace the lid quickly.

Then follow steps **11** to **14** using filter paper discs soaked in appropriate solutions for the three control plates. With the paper disc method you can place all four discs on one agar plate.

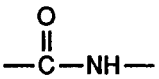
Disposal

Used cultures, syringes and plates must be sealed inside an autoclavable plastic bag (or a roasting bag) and sterilised before disposal.

Comments

a The 6-APA is obtained from penicillin G which is produced naturally by a mould.

b The sodium salt of 6-APA is more soluble in water than the free acid.

c HCl (→ NaCl + H_2O); amide bond 

d The crude reaction mixture is first acidified. This releases the free acid form of the penicillin, which is non-ionic and more soluble in ethyl ethanoate than in water. It is extracted into ethyl ethanoate to remove it from water-soluble impurities, such as HCl and NaCl, and unreacted 6-APA. The second extraction with ethyl ethanoate removes the last traces of the penicillin from the aqueous layer. The acid form is then converted back to its sodium salt by adding sodium hydrogencarbonate solution. This is ionic and can be extracted back into water.

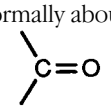
Note

It is important to add ethyl ethanoate to the reaction mixture *before* the aqueous phase is acidified. The penicillin carboxylic acid formed on acidification is then removed immediately from the acidic aqueous phase by dissolving in the 'neutral' organic phase.

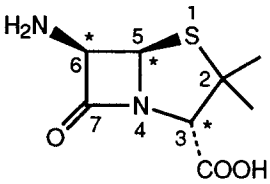
e To show that any bacterial activity observed is due to the penicillin and not to either of the starting materials or substances present in the nutrient agar.

MD5.2 A closer look at the structure of penicillins (optional extension)

a Amide group

b The tetrahedral bond angle around a saturated carbon atom is normally about 109°. The C–C–C bond angle in the group  is about 120°.

The bond angles in the β-lactam ring are 90°, and so the ring is very strained. It reacts readily to give strain-free open-chain compounds which are more stable.

c 

Three chiral carbon atoms (*)

d Changes to the stereochemistry around the chiral centres change the shape of the molecule, so that it no longer fits well onto the active site of the bacterial enzyme. Students can use their models to investigate this effect.

Note

In fact, penicillin has a dipeptide structure, which resembles that of two amino acids in the polypeptide chains present in bacterial cell walls. The stereochemistry around carbon-3 has the D-configuration and resembles D-alanine. This is the amino acid the cross-linking enzyme links on to.

e The synthesis is complex and yields are low. It is difficult to achieve the correct stereochemistry at each chiral centre. The process is uneconomical compared to using 6-APA obtained from moulds.

f Natural penicillins are made entirely by the mould with the appropriate side-chains attached. Semi-synthetic penicillins are synthesised from 6-APA, which is obtained by enzyme hydrolysis of a naturally produced penicillin.

g **i** Methicillin and flucloxacillin are resistant to attack by the β-lactamase enzyme. They both have very bulky side-chains. In each case, the C atom attached directly to the  group in the side-chain is completely substituted.

ii Bulky side-chains make a profound difference to the overall size and shape of the molecule. This prevents a good fit of the structure onto the β-lactamase active site. (Students could convert their model of penicillin V into methicillin to investigate this effect.)

iii Methicillin and flucloxacillin fit onto the active site of the cell wall enzyme, but not onto that of β-lactamase. The β-lactamase site may therefore be smaller.