AMBROX

Development of a two-step “green” synthesis for (-)-ambafuran production

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our future through science

Outline of the presentation

- Ambra and Ambrox
- Sclareol from Clary sage
- Chemical steps in the production of the diol intermediate
- Ambrox commercial synthesis from sclareol
- Problems with the synthetic route
- Bioconversion of sclareol
- Objectives of the project
- Biocatalysis
- The new CSIR process
- Production of the intermediate
- Conversion to (-) ambrox
- Advantages and competitive edge
- Questions

Ambra (Ambergris)

- Ambra -pathological metabolite of the sperm whale
- Ambra - arise from injuries in the whale intestines as a result of certain food intakes
- Excreted chunks of ambra are exposed to sunlight and air at the surface of the sea
- A number of oxidation products with a pronounced odour are gradually formed

Ambra (continued)

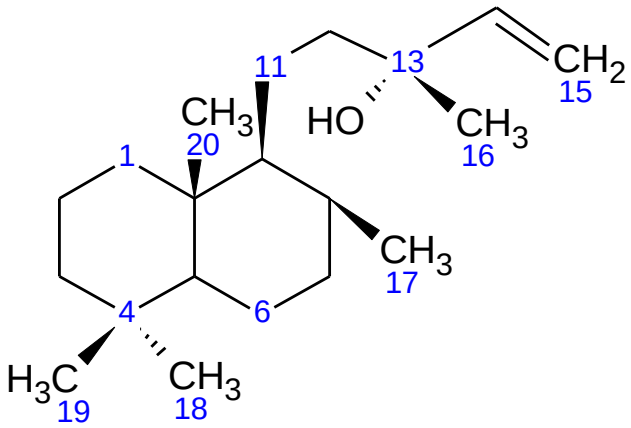
- Highly valued as a sensualizing fixative in perfumery
- Due to its scarcity, it is a rare item on the perfumer's shelf
- One of the most important ambra odorants is ambrox
- Today it is synthesized from the diterpene sclareol
- Sclareol found in the plant Clary sage
- The powerful and elegant odor of ambrox matches the first four tonalities of aged ambergris tincture:
  - ✓ wet mossy forest soil
  - ✓ strong tobacco
  - ✓ balsamic sandalwood
  - ✓ warm animal musk

Ambrox background

- (-)-Ambrox® - moist, soft, creamy, persistent, warm, animalic, amber odor with a velvety effect
  Smell threshold = 0.3 ppb
- (+)-Ambrox® - accentuated woody note and lacks the strong "animalic warmth" of the (-)-isomer
  Smell threshold = 2.4 ppb
- (-) Ambrox is recognised as the prototype of all ambergris odorants
- The price of (-) Ambrox is quite high ($800/kg)
- Several synthetic routes have been developed for (-) Ambrox and its racemate
- Commercially (-) Ambrox is made from **sclareol** in 8 steps

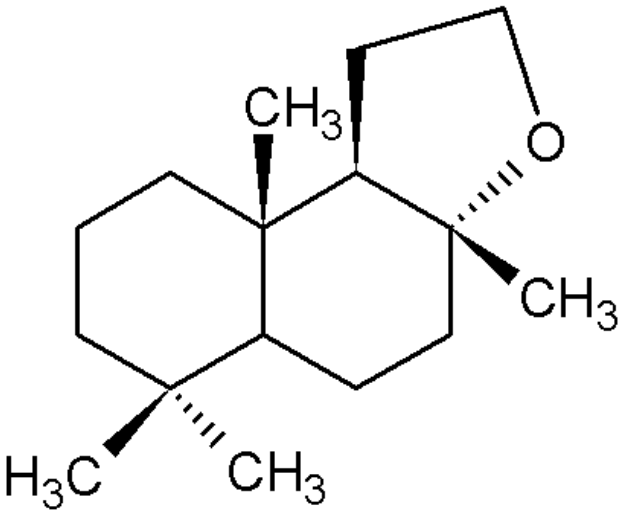
Sclareol

- Sclareol is extracted from *Salvia sclarea*
  - EtOH followed by solvent evaporation
  - Boiling of the residue with a solution of KOH in EtOH
- The distilled essential oil is used as a fragrance and has a "sweaty", spicy, or "hay-like“ smell

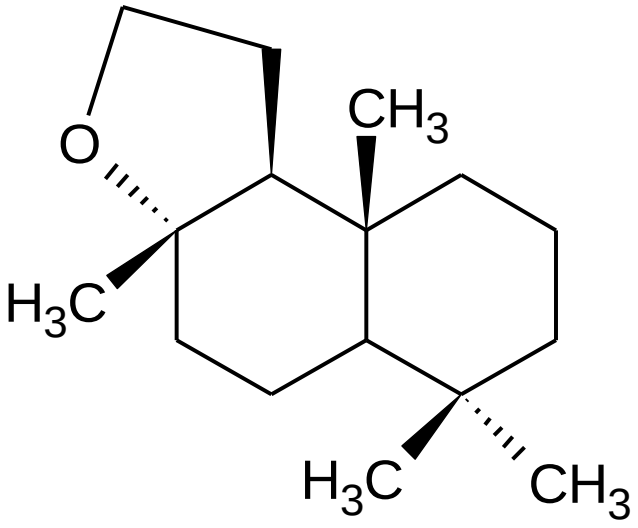


Sclareol

Ambrox

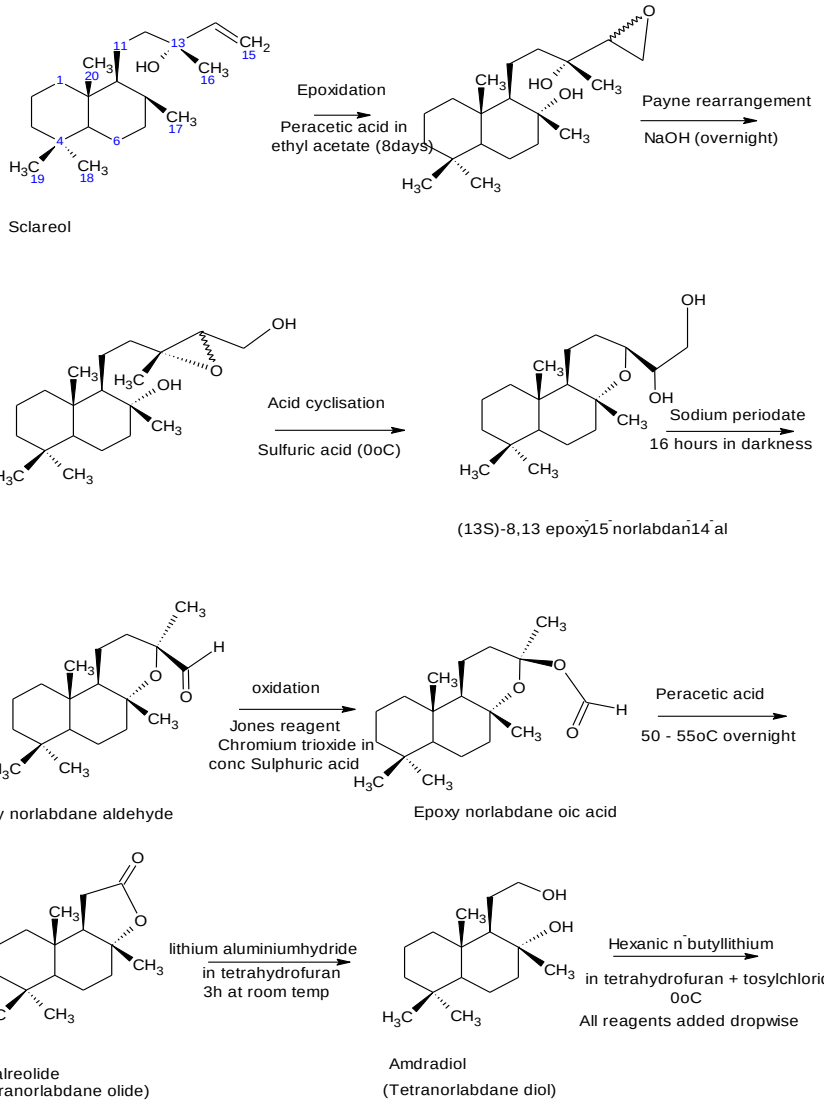


(-) Ambrox

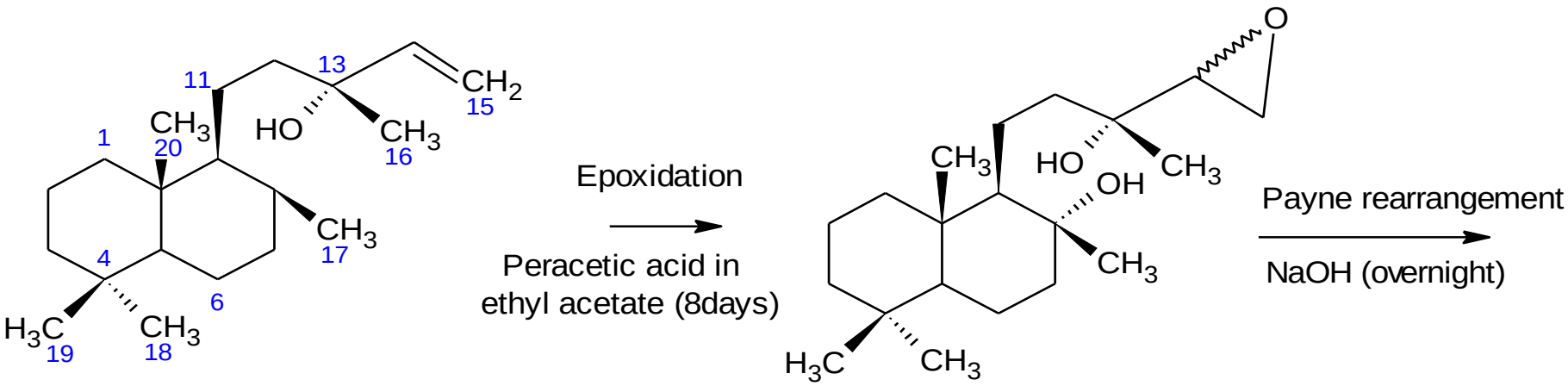


(+) Ambrox

Chemical Steps to produce the diol intermediate

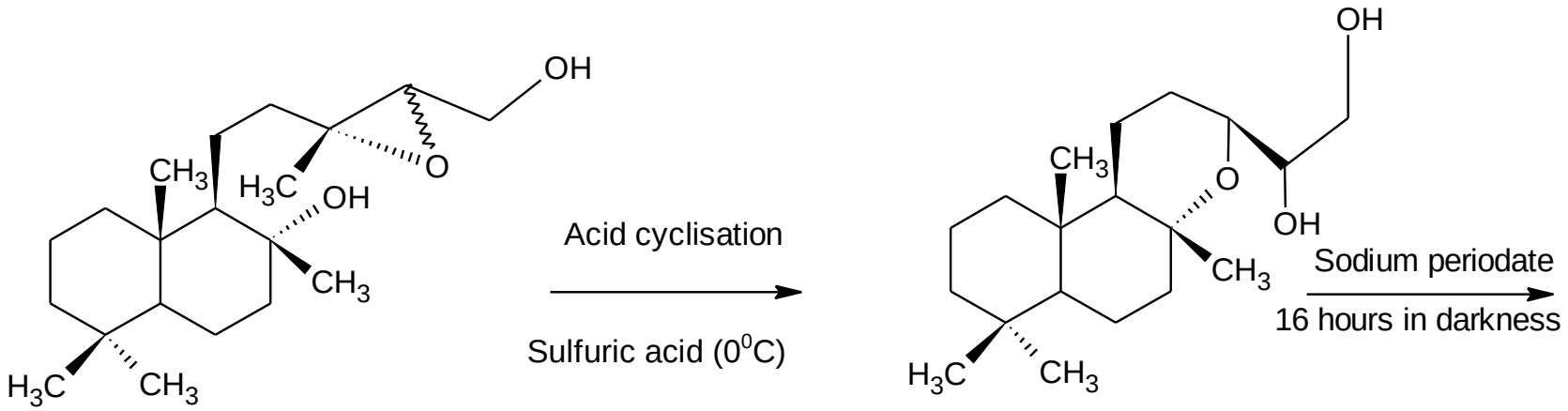


Ambrox commercial synthesis from sclareol



Sclareol

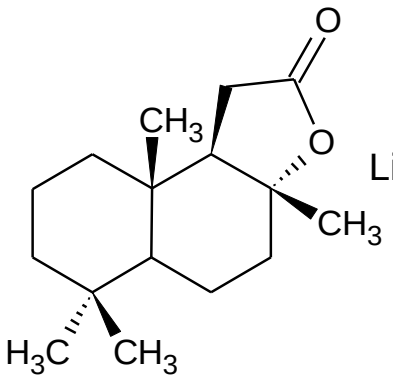
Ambrox commercial synthesis from sclareol



(13S, 14RS)-8,13 epoxylabdane, 14,15 diol

(13S)-8,13 epoxy -15-norlabdan -14-al

Ambrox commercial synthesis from sclareol



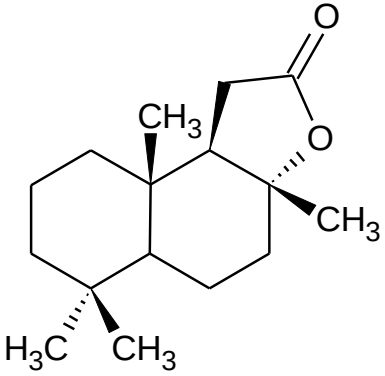
Sclareolide

(Tetraorlabdane oxide)

Lithium aluminiumhydroxide

in THF

3h @ room temp

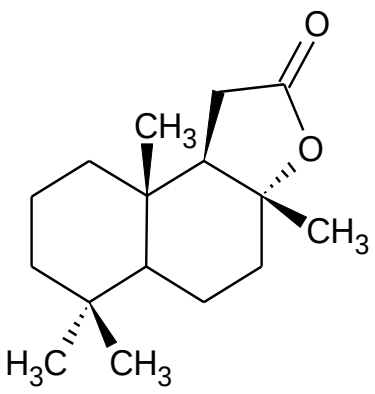


Epoxy norlabdaneoic acid

Peracetic acid

50 - 55oC overnight

Ambrox commercial synthesis from sclareol



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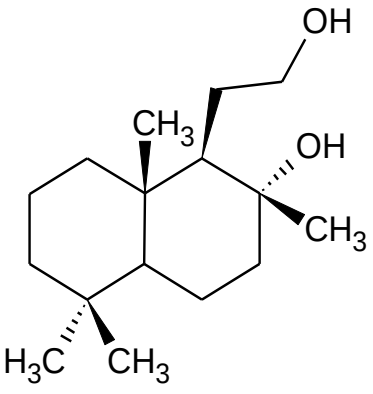


Amdradiol

(Tetraorlabdane diol)

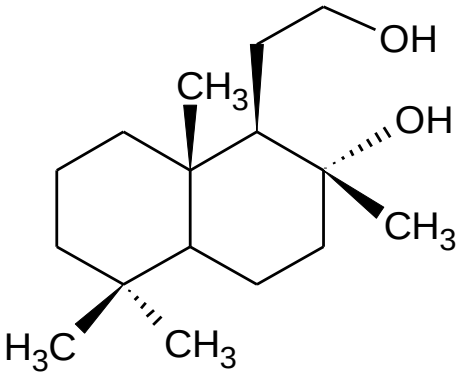
Hexanion butyllithium

in THF + tosylchloride

0 ^{0}C

All reagents added dropwise

Ambrox commercial synthesis from sclareol



Amdradiol

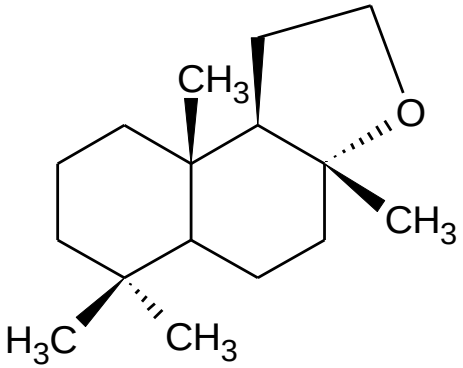
(Tetranorlabdane diol)

Hexanic n-butyllithium

in tetrahydrofuran + tosylchloride

0^0C

All reagents added dropwise



(-) Ambrox

Problems with synthetic route

- The first reaction takes 8 days
- Generation of waste
  - has to be disposed of in special manner
- Byproducts are formed
- Use of harsh chemicals such as:
  - Butyllithium in hexane

    ✓ Dangerous chemical - water-reactive and extremely flammable
    ✓ Extremely corrosive and causes severe burns
    ✓ On inhalation may cause chemical pneumonitis which may be fatal
    ✓ Chronic exposure to fumes may cause kidney damage

Problems with synthetic route (continued)

- Tosyl chloride
  - ✓ Contact with water liberates toxic gas
  - ✓ Causes burns by all exposure routes
  - ✓ Serious effects on respiratory system
  - ✓ Reaction has to be done at 0ºC
  - ✓ Reagents have to be added dropwise to control reaction
- The last cyclodehydration reaction can also be done with tosylchloride in pyridine
  - ✓ The pyridine is a toxic chemical which can cause liver damage and liver cirrhosis
  - ✓ The pyridine has an effect on the final fragrance

Problems with synthetic route (continued)

- Pyridine can be replaced by sodium hydride and sodium tert-amyl alcohol
- Sodium hydride is very dangerous when wet
- It is extremely caustic
- Reacts violently with water and forms a flammable and explosive gas (hydrogen)

The above problems will be aggravated on large scale implementation with severe health risks for the operators

Bioconversion of sclareol

- Organic synthesis
  - nature identical flavours available at a cheap price
- Changes in lifestyle
  - dictating consumer demand for new flavours and fragrances
  - more environmentally friendly processes
- Natural flavours and fragrances
  - prepared by biotechnological routes
  - alternatives to the time-consuming and expensive
  - extraction from botanical sources

Objectives of the project

- The main objective

  ✓ find an alternative synthesis of (-) Ambrox from sclareol
  ✓ using a bioconversion or biocatalysis route
  ✓ it must result in a natural or nature-identical product

- The final aim

  ✓ scale the process to bench scale
  ✓ finalise a technology package which the client can use to commercialise the technology

Biocatalysis

Biocatalysis can be defined as utilization of natural catalysts, such as protein enzymes, to perform chemical transformations on organic compounds. Both enzymes that have been more or less isolated or enzymes still residing inside living cells are employed for this task.

History of Biocatalysis

- Throughout the history of mankind
  - microorganisms have been of enormous social and economic importance
- Microorganisms can modify certain compounds
  - by simple, chemically well defined reactions
  - catalysed by enzymes
- These processes are now either called biotransformations or biocatalysis
- Many biotranformation type reactions possible to replace chemical type reaction

Advantages of Biocatalysis

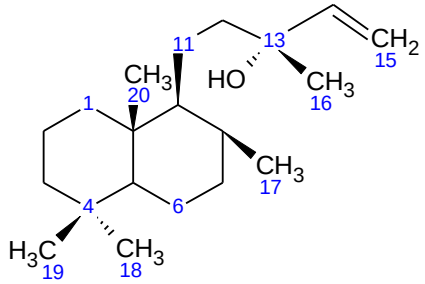
- Biocatalysis is a relatively green technology
- Enzyme reactions can be carried out:
  - in water
  - at ambient temperature
  - neutral pH
- There is no need for:
  - high pressure
  - extreme conditions
  - save energy normally required by chemical processes
- The waste streams are normally more environmentally friendly

Advantages of Biocatalysis (continued)

- Biocatalysis is a useful supplementary technology for chemical industry
- The enzymes can catalyse some stereoselective synthesis
  - may not be easily conducted by classical organic chemistry
- Biocatalysis can sometimes replace several chemical steps
- Enzyme reactions are also conducted in organic solvents
  - substrates which do not dissolve in water

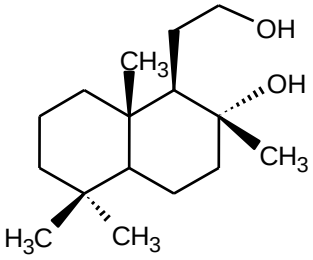
New CSIR process

New CSIR process



Sclareol

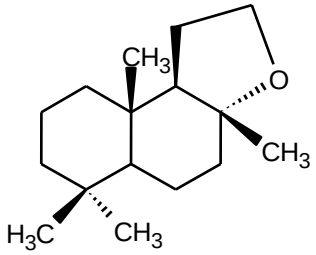
Hyphozyma roseoniger



Amdradiol

(Tetranorlabdane diol)

Zeolites



(-) Ambrox

Production of the intermediate diol

- *Hyphozyma roseoniger* was purchased from ATCC
  - patent has expired and the company does not exist anymore
- Screening for new microorganisms
  - Soil samples were collected from various places
- Microorganisms grown on agar containing the sclareol as carbon source
- Produced the intermediate diol
- Optimisation of *Hyphozyma roseoniger*
  - ✓ *Different media compositions*
  - ✓ *Temperature profiles*
  - ✓ *Substrate concentrations*
  - ✓ *Addition of surfactant*
  - ✓ *Induction of activity*

Production of the intermediate diol (continued)

- Very simple minimal medium
- Induction with low initial substrate
- High initial concentrations of the sclareol resulted in a lag phase before conversion
- Full conversion from sclareol to intermediate diol at concentrations up to 15 g/L
- Conversion takes place during active growth of microorganism
- Optimum temperature at 20°C
- Unwanted by-products start to form at temperatures above 25°C

Production of the intermediate diol (continued)

- The identity of the intermediate diol was confirmed by LC-MS analysis
- The diol was then extracted into organic solvent
- Solvent was evaporated under reduced pressure
- Dried product is dissolved in hexane

Conversion to (-) Ambrox

- Different strategies were tested to convert the intermediate diol to (-) Ambrox
- An aldolase enzyme known to catalyse cyclisation
- Chemical methods for cyclodehydrations tested (eg. DMSO)
- A novel method was discovered for the cyclodehydration of the intermediate diol to (-) Ambrox
- New method uses dried zeolites
- 100% conversion from the intermediate to the (-) Ambrox
- The chirality of the (-) Ambrox was confirmed on GC

Conversion to (-) Ambrox (continued)

- Initially zeolites were dried at 500°C under vacuum at WITS University
- Currently it is dried with a conventional household microwave oven
- The conditions of the conversion are very mild
- The reaction takes place at room temperature
- The reaction is complete in 2 – 5 hours
- The catalyst can be reactivated and re-used
- The product is obtained through removal of the catalyst via a simple filtration and evaporation of the solvent

Advantages of the new process

- Current industrial method uses 8 steps to the final product
- The new process is complete in 2 steps
- The chemical method uses harsh chemicals and conditions
- At least two of the chemical reactions are done at 0ºC because of the violent reactivity and by-products forming at higher temperatures
- The processes at low (0ºC) as well as reactions at higher temperatures during the chemical reaction requires high energy input
- The waste streams from the chemical process requires special consideration for disposal

Advantages of the new process **(continued)**

- The waste streams from the new process is environmentally acceptable
- In the new process, the solvent for extraction can be re-used following distillation
- No harsh chemicals with possible effects on human health are used in the new process
- The new process has been patented

Competitive edge for the Industrial partner

- The Industrial partner is specialising in flavour and fragrances
- The product will add to the basket of products
- The process will be considered “green”
- This product will be unique in South Africa
  - The industrial partner will be the sole natural producer
  - One of possibly 3 producers worldwide
- Clary sage will be grown for production of the starting material for the ambrox product
  - Job creation

Acknowledgements

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- THRIP (for financial assistance)
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CSIR for infrastructure and evaluation of the Intellectual Property

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Thank You