Quality of active pharmaceutical ingredients and industrial bioprocess;

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European Pharmacopoeia (Council of Europe Strasburg): member of the commission for "Certification Procedure for the chemical purity and microbiological quality evaluation".

IBC: President of the Italian Biocatalysis Center

“Qualità dei prinicipi attivi: concetti generali”

Quality of Active Pharmaceutical Ingredients (API)

New API :

EMEA (ICH Guide line)

Generic API:

ICH Guide line

Farmacopea (EP, USP, JP)

Control of Quality of API

Obtained by chemical process

1. **Impurities in New Drug Substances** (ICH Q3A R2)
2. Ph. Eur. General Monograph 2034, Substances for Pharmaceutical Use
3. **Impurities testing** (ICH Q3A); Ph. Eur. General Monograph 2034, Substances for Pharmaceutical Use)
4. **Residual Solvents** (ICH Q3C); Ph. Eur. general text 5.4
5. **Specific Monographs (**Ph. Eur. **)**

Product obtained by Fermentation Process

1. General Monograph for **Product of Fermentation 01/2008:1468;**
2. **Specific Monographs (**Ph. Eur. **)**

General monograph (2034)

Related substances. Unless otherwise prescribed or justified and authorised, organic impurities in active substances are to be reported, identified wherever possible, and qualified as indicated in Table 2034.-1.

Table 2034.-1. – *Reporting, identification and qualification of organic impurities in active substances*

Use	Maximum daily dose	Reporting threshold	Identification threshold	Qualification threshold
Human use or human and veterinary use	≤ 2 g/day	> 0.05 per cent	> 0.10 per cent or a daily intake of > 1.0 mg (whichever is the lower)	> 0.15 per cent or a daily intake of > 1.0 mg (whichever is the lower)
Human use or human and veterinary use	> 2 g/day	> 0.03 per cent	> 0.05 per cent	> 0.05 per cent
Veterinary use only	Not applicable	> 0.1 per cent	> 0.2 per cent	> 0.5 per cent

Specific thresholds may be applied for impurities known to be unusually potent or to produce toxic or unexpected pharmacological effects.

If the individual monograph does not provide suitable control for a new impurity, a suitable test for control must be developed and included in the specification for the substance.

The requirements above do not apply to biological and biotechnological products, peptides, oligonucleotides, radiopharmaceuticals, products of fermentation and semi-synthetic products derived therefrom, to crude products of animal or plant origin or herbal products.

Peptides

- Generally used at low daily dose
- Many potential related substances: isomers, results of failures in sequences, cleavages or coupling
- Requirements for related substances included in the general monograph 2034 from 1/07/2009:

Table 2034.-2. – *Reporting, identification and qualification of organic impurities in peptides obtained by chemical synthesis*

Reporting threshold	Identification threshold	Qualification threshold
> 0.1 per cent	> 0.5 per cent	> 1.0 per cent

1. Fermentation Processes

01/2008:1468

PRODUCTS OF FERMENTATION

Producta ab fermentatione

This monograph applies to indirect gene products obtained by fermentation. It is not applicable to:

- *monographs in the Pharmacopoeia concerning vaccines for human or veterinary use;*
- *products derived from continuous cell lines of human or animal origin;*
- *direct gene products that result from the transcription and translation from nucleic acid to protein, whether or not subject to post-translational modification;*
- *products obtained by semi-synthesis from a product of fermentation and those obtained by biocatalytic transformation;*
- *whole broth concentrates or raw fermentation products.*

General monograph « Products of fermentation » (1468)

- Scope:
  - « Indirect » gene products obtained by fermentation
- Out of the scope:
  - Vaccines, products from continuous cell lines, of animal/human origin
  - Direct gene products
  - Semi-synthetic products, biocatalytic process
- Provides general requirements for manufacture of fermentation products. Compliance mandatory for these products

FERMENTATION

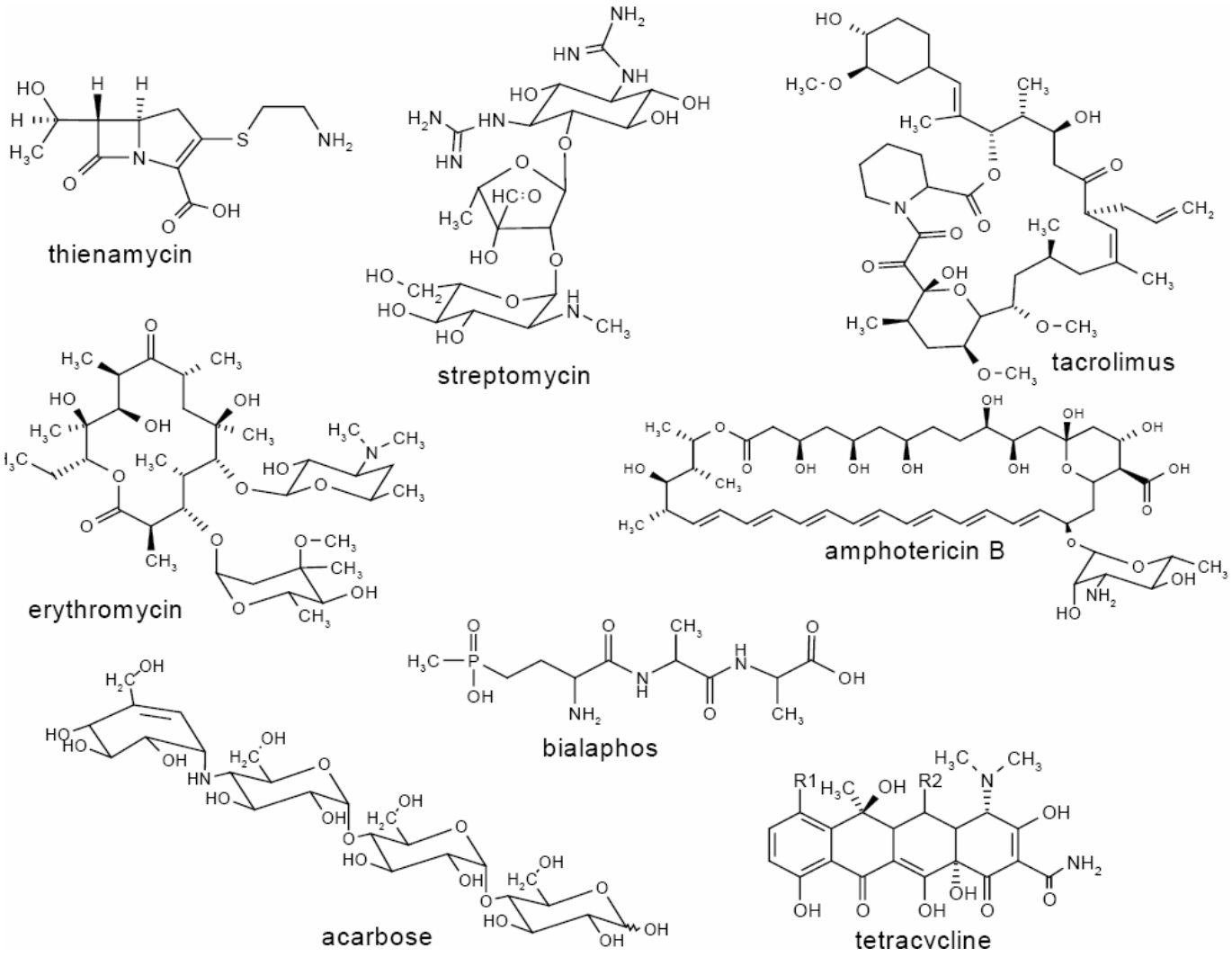
Advantages

- One step reaction: synthesis of complex molecules
- Low costs

Disadvantages

- Complex mixtures
- Contaminants with different structures
- Residuals arising from micro-organisms
- Complex downstream

Product of fermentation Complex structures



Simple compounds, normally prepared by chemical synthesis, are more and more prepared by fermentation.
For example:

- Aminoacids
- Nucleosides and nucleotides
- Vitamins

Control of the quality is depending from the process used.

Specificities of fermentation products

- Variety of possible processes
- A biosynthetic process may be more difficult to control than a chemical process
- May lead to complex mixtures
- Need to have powerful separation techniques for the control of related substances

Requirements

- Source and history of the producer micro-organism
- Characterisation, stability, of the producer micro-organism
- Detailed description of fermentation, incl materials, preparation of media, downstream processing
- In-process controls at all stages
- Purity of the final substance: impurity profile and specification

Impurity profile

- Impurities (3.2.S.3)
  - Describe all potential related substances (sometimes complex), focus on actual impurities
  - Address residual solvents according to European guidelines
  - Discuss particular impurities arising from fermentation: residues of substrates, cells residues, proteins,…

Limits for related substances

- Set limits according to the monograph
- Set limits for the other known impurities
  - Qualify impurity profile by comparison with products already on the market, or tox data
  - In line with levels found in batches
- Set limits for unknown impurities + total impurities

Limits for residual solvents

- According to ICH and CHMP guidelines on Residual solvents
- Show absence of the solvents used during the process before purification
- Set limits and propose methods for solvents used during purification

Other impurities

- Demonstrate that there are no residues from fermentation
- Proteins: not a concern for oral use (a lime proposed), to be addressed if parenteral use (absence should be demonstrated)
- DNA
- The Ph. Eur provides general methods which can be used

downstream:
Elimination of residuals from micro-organism

- **Extraction with organic solvents**

allows complete elimination of residuals. Presence of residual indicate a not well separation of phases.

- **Crystallization in organic solvent**

Most adequate for residuals elimination

- **Ultra filtration**

Residuals of low molecular weight proteins and peptides

- **Chromatographic columns**

Complete elimination of protein is ensured depending from the chromatographic conditions

Problems for high water soluble products:

- Peptides
- Amino acids
- Oligosaccharides
- Nucleosides and nucleotides

Evaluation of residuals arising from the micro-organism

- Absence of residuals from the microorganism should be demonstrated during process validation
- Product for injection should be carefully controlled

Nucleic acids

- Absorbance tests
- RT-PCR or PCR

Proteins

- Colorimetric tests: Bradford, Lowry or other according to the *Monograph for Assay of Total Protein 2.5.33* (01/2008:20533)
- Electrophoresis

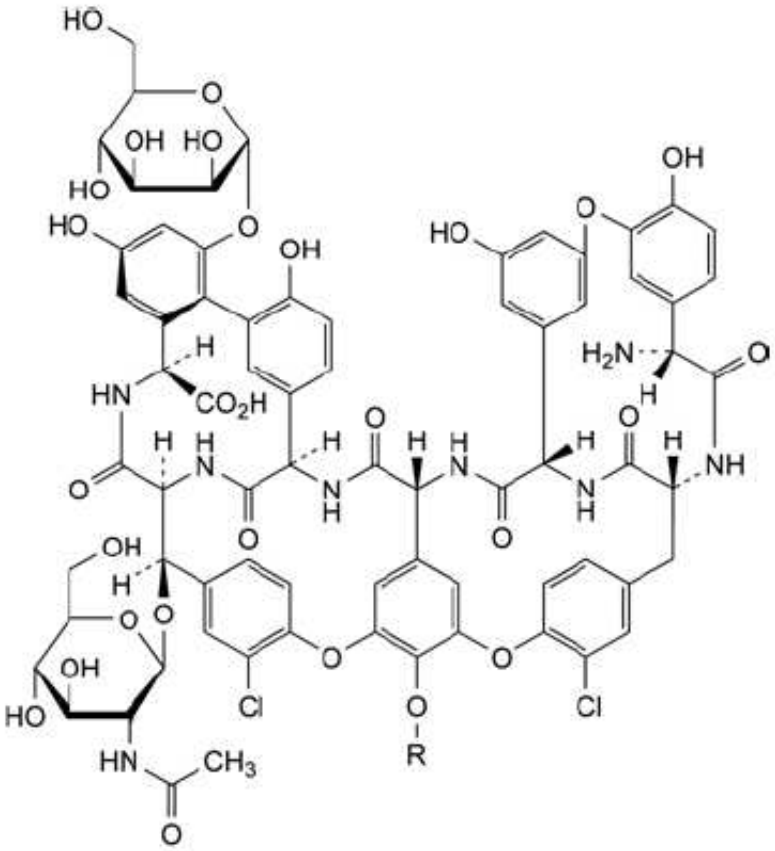
The specific monographs

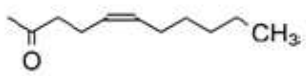
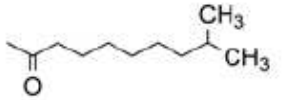
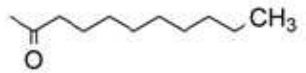
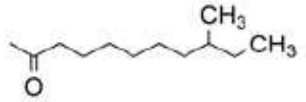
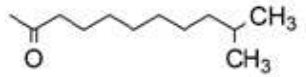
- Cover the quality of individual substances. Eg. Erythromycin,..
- Compliance mandatory in Ph. Eur member states
- Take into account the specificity of fermentation products
- Reflect the profiles of products approved for the European market
- Transparency list: describes impurities detected by the methods of the monograph

Teicoplanine

01/2009:2358
corrected 6.6

Teicoplaninum



Teicoplanin	R	R'
A_{2-1} $C_{88}H_{95}Cl_2N_9O_{33}$ M. W.: 1878		
A_{2-2} $C_{88}H_{97}Cl_2N_9O_{33}$ M. W.: 1880		
A_{2-3} $C_{88}H_{97}Cl_2N_9O_{33}$ M. W.: 1880		
A_{2-4} $C_{89}H_{99}Cl_2N_9O_{33}$ M. W.: 1894		
A_{2-5} $C_{89}H_{99}Cl_2N_9O_{33}$ M. W.: 1894		
A_{3-1} $C_{72}H_{68}Cl_2N_8O_{28}$ M. W.: 1564	H	

Is a Complex mixture of products. Problems related to:.

- Identification
- Quality

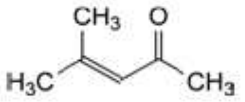
Angtibiotics: Teicoplanine

Limits:

- *teicoplanin A_2 group*: minimum 80.0 per cent;
- *teicoplanin $A_{2\text{-}2}$*: 35.0 per cent to 55.0 per cent;
- *teicoplanin $A_{2\text{-}1}$ group*: maximum 20.0 per cent;
- *teicoplanin $A_{2\text{-}3}$ group*: maximum 20.0 per cent;
- *teicoplanin $A_{2\text{-}4}$*: maximum 20.0 per cent;
- *teicoplanin $A_{2\text{-}5}$ group*: maximum 20.0 per cent;
- *teicoplanin A_3 group*: maximum 15.0 per cent;
- *total of impurities other than mesityl oxide with a relative retention more than 1.25*: maximum 5.0 per cent;
- *disregard limit*: the area of the peak due to teicoplanin $A_{2\text{-}2}$ in the chromatogram obtained with reference solution (b) (0.25 per cent).

IMPURITIES

Specified impurities: A.



A. 4-methylpent-3-en-2-one (mesityl oxide).

Composition of the "Complex"
and related substances are strictly dependent from:

- Microorganism
- Control of the Fermentation process
- Raw material used for fermentation process

Limit for impurities?

Draft: Guide line on setting specification for related impurities in antibiotics

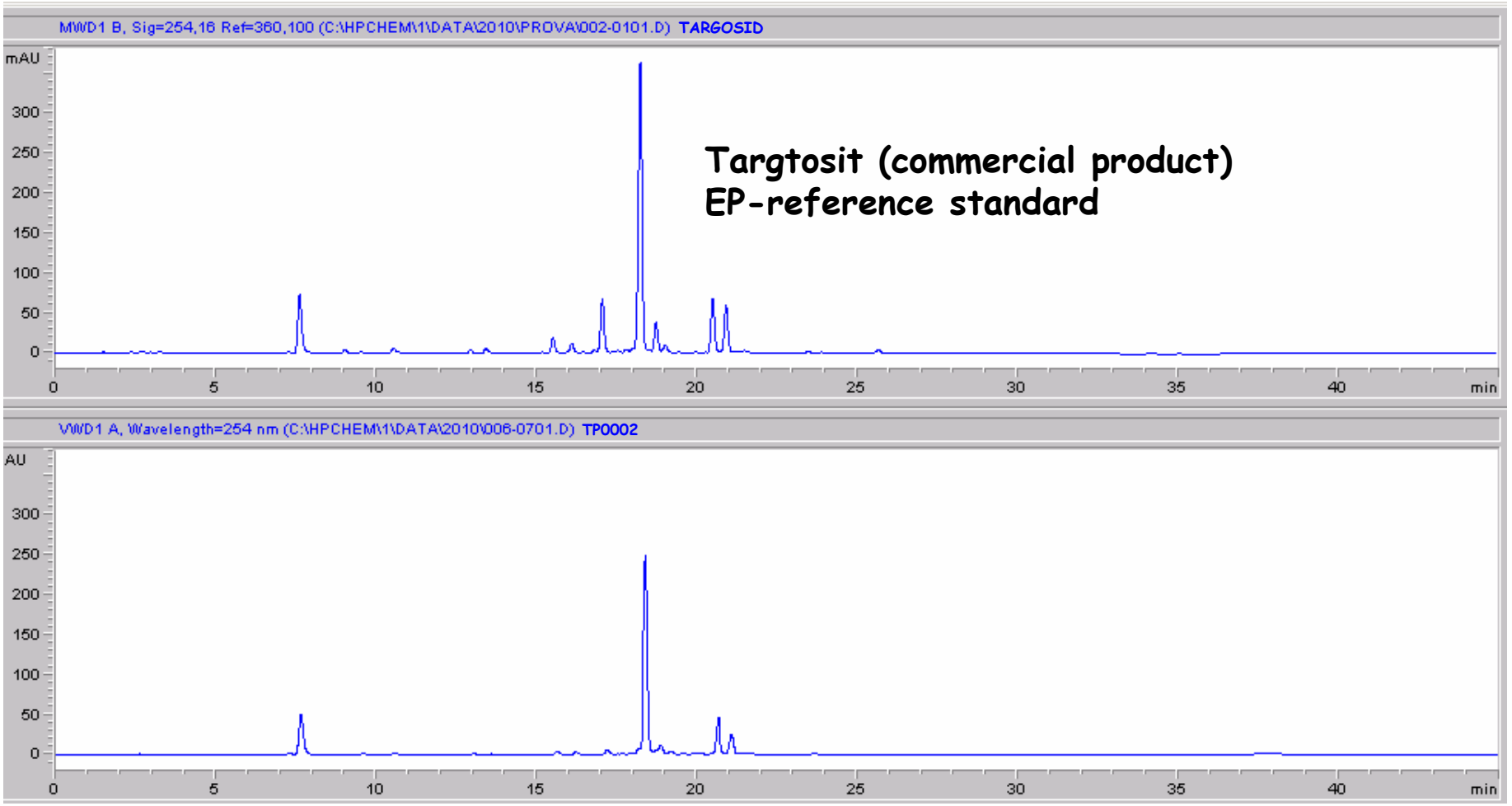
14 July 2010: EMA/CHMP/CVMP/QWP 199250/2009
End of Consultation 31 January 2011

Proposed limits for Antibiotics manufactured by fermentation: Fanmily of compound

Reporting threshold:	0.10%
Identification threshold	0.15%
Qualification threshold unknown	0.15%
Qualification threshold related	**0.50%**

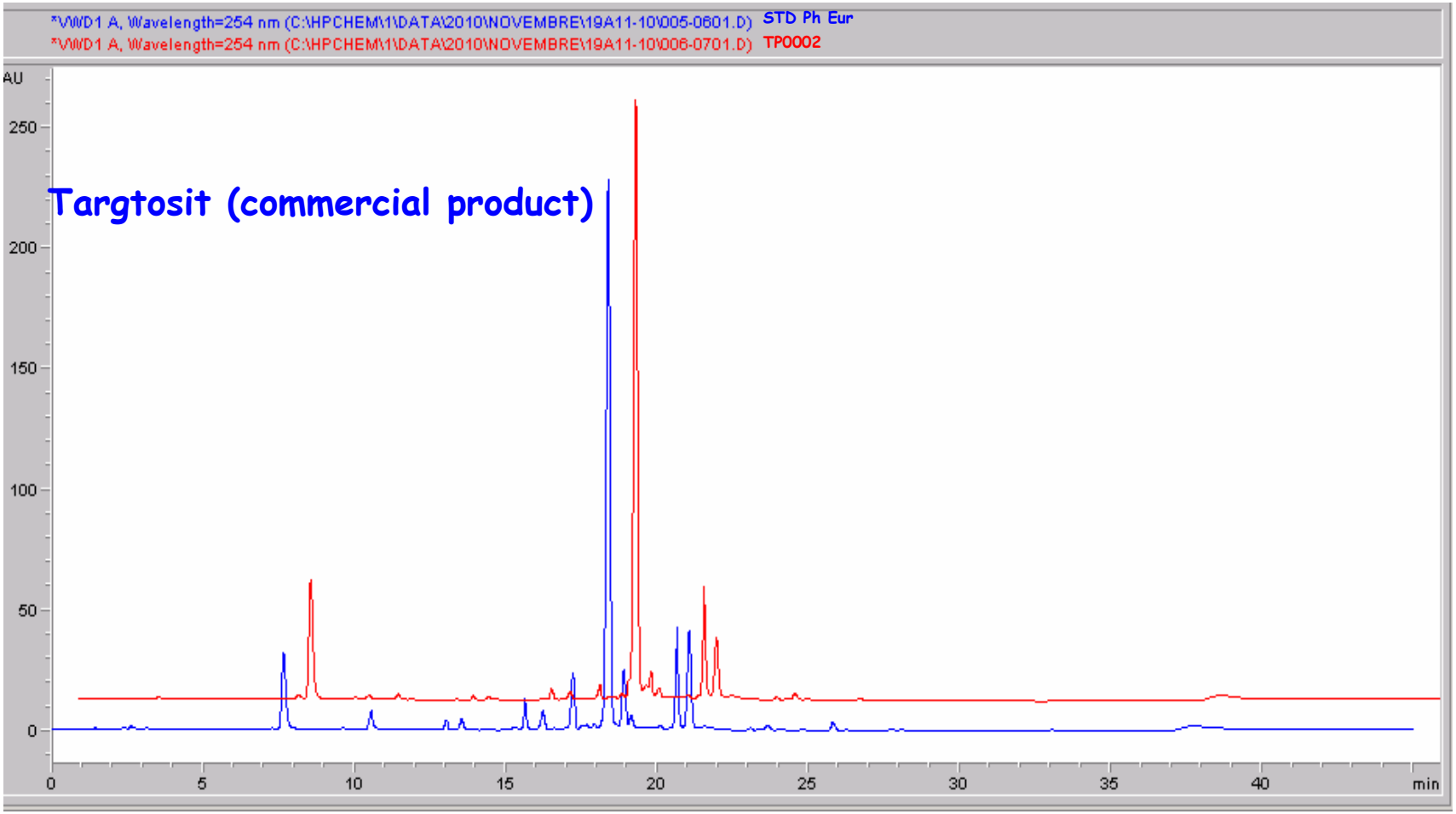
Identification at least by HPLC-MS

Teicoplanine Producer 1



Pattern and related substances conform with the EP-CRS

No new impurities detected


*\WD1 A, Wavelength=254 nm (C:\HPCHEM\1\DATA\2010\NOVEMBRE\19A11-10\005-0601.D) STD Ph Eur
*\WD1 A, Wavelength=254 nm (C:\HPCHEM\1\DATA\2010\NOVEMBRE\19A11-10\006-0701.D) TP0002
Targtosit (commercial product)
AU
250
200
150
100
50
0
0
5
10
15
20
25
30
35
40
min

Batch Analysis

Test	Specs	TARGOSID	TP0002
A2 Group	**minimum 80,0%**	87,9	86,8
A2-1	**maximum 20,0%**	17,1	6,1
A2-2	**35,0 - 55,0 %**	45,1	54,7
A2-3	**maximum 20,0%**	7,7	7,6
A2-4	**maximum 20,0%**	8,8	10,9
A2-5	**maximum 20,0%**	9,1	7,5
A3 Group	**maximum 15,0%**	9,6	11,4
Total Impurities	**maximum 5,0%**	2,5	1,8

Teicoplanine Prodiucer 2: Japan Pharcopoeia (JP)

JP Specification	Teico Batch A	Teico Batch B	Teico Batch C
TA2 Group NLT 80%	95.24	94.04	90.50
TA3 Group NMT 15%	3.71	4.45	8.28
Other NMT 5%	1.05	1.51	1.22

Commercial Medicinal product (Tangosit) and the new Teicoplanine API meet specification for JP

Csilla FranK; Controllo della qualità di un prodotto di fermentazione: aspetti tecnici e regolatori
Thesis "Master in Discipline Regolatorie 2008"; University of Pavia; Pavia; Italy

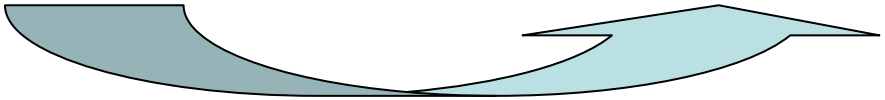
Teicoplanine: EP Specification

Out of Specification

EP Specification	Teico Batch A	Teico Batch B	Teico Batch C
TA2-1 Group NLT 20%	10.65	6.32	8.62
TA2-2 Group NMT 35-55%	**59.54**	**56.54**	**55.01**
TA2-3 Group NMT 20%	9.04	10.69	8.95
TA2-4 Group NMT 20%	4.27	7.51	5.05
TA2-5 Group NMT 20%	2.79	5.26	3.57

Conform with the Specification

EP Specification	Teico Batch A	Teico Batch B	Teico Batch C
TA2-1 Group NLT 20%	9.3	8.3	7.7
TA2-2 Group NMT 35-55%	**47.7**	**47.7**	**48.7**
TA2-3 Group NMT 20%	8.2	7.5	7.0
TA2-4 Group NMT 20%	14.8	14.1	13.5
TA2-5 Group NMT 20%	6.7	7.1	7.1



Modulation of fermentation condition
Selection of the ingredient used during fermentation

Csilla FranK; Controllo della qualità di un prodotto di fermentazione: aspetti tecnici e regolatori
Thesis "Master in Discipline Regolatorie 2008"; University of Pavia; Pavia; Italy

Example of fermentation product: L-serine

Isolation from fermentation broth:

- Filtration of biomass
- Anionic and cationic exchange resins

Purification:

- Ultrafiltration of water solution (cut-off m.w.>6000)
- Crystallization from water

Possible impurities:

Aminoacids. Analysis performed with A.A. analyzer (each NMT 0.2%; total NMT 1.0%).

Absence of protein demonstrated by Bradfford assay (colorimetric: LOD 1ug/mL)

2. Enzymatic Bioprocesses

Different "catalysts" can be used

- **Cell paste (wool cell)**
- **Fermentation Broth**
- **Crude extract**

True Fermentation

- Possible Residuals arising from the ` micro-organism
- Complex downstream

- **Purified Enzymes**
- **Solid or Immobilised Enzymes**

Chemical products

- Potential Residual Proteins
- No other residual from fermentation

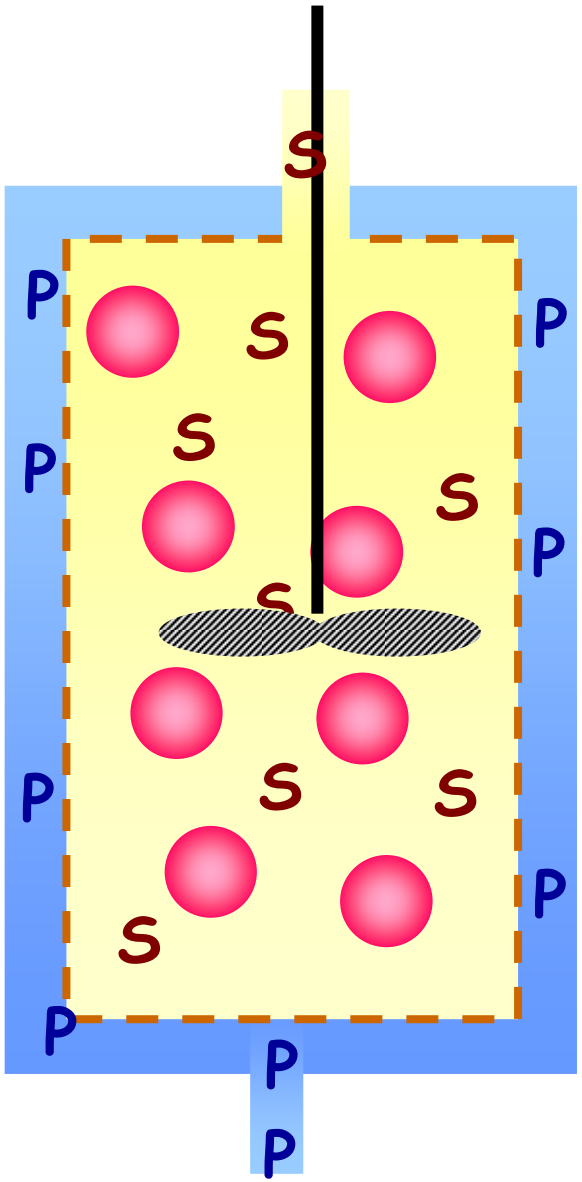
USE OF ISOLATED ENZYMES IN THE SYNTHESIS OF ACTIVE INGREDIENTS

ENZYME PREPARATION

1. Free soluble enzymes

2. Enzymes immobilized on solid supports

1. FREE SOLUBLE ENZYMES

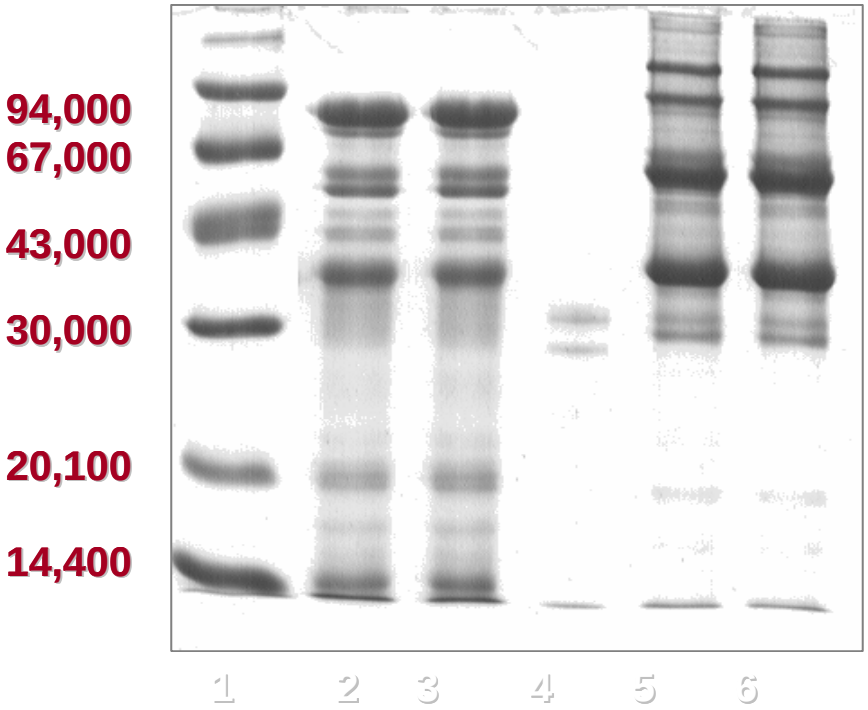


Downstream:

Separation of the enzyme from the product

- REACTORS WITH ULTRAFILTRATION
- EXTRACTION

CRUDE EXTRACT ARE OFTEN COMPLEX
Mixture of several proteins



SDS-PAGE RML

- Contamination by different proteins depending from filter porous size (cut-off)
- Side reactions catalyzed by different enzymes
- Other contaminants contained in the crude extract

Example of Enzymatic bioprocess: L-serine

Enzyme Isolation from fermentation broth:

- Filtration of biomass
- Solution is directly used without purification of the enzyme

Purification:

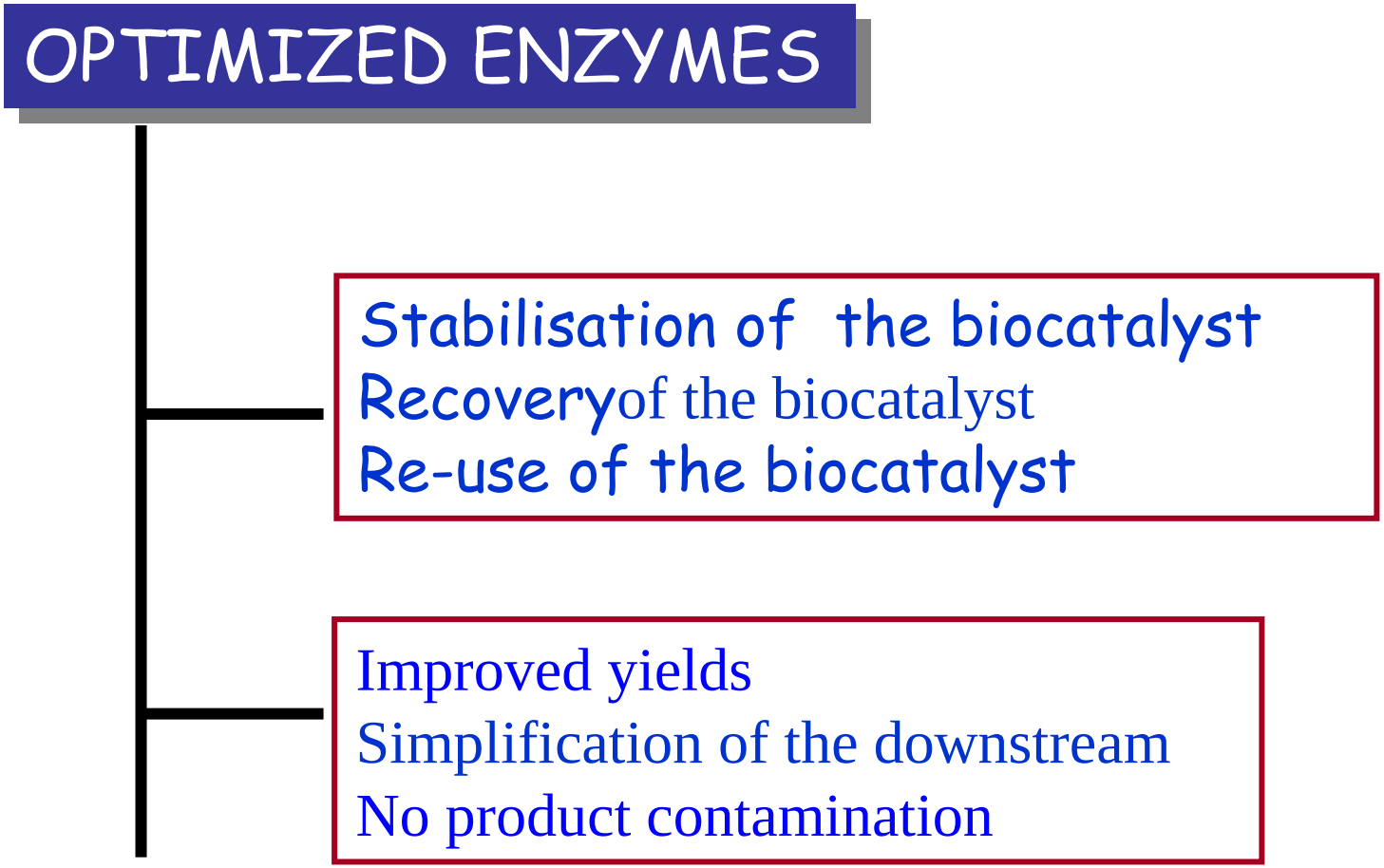
- Ionic exchange colums
- Crystallization from organic solvent/water solution
- Ultrafiltration of water solution (cut-off m.w.>5000)
- Crystallization from water

Possible impurities:

Glicine (starting material) and other A.A. Analysis performed with A.A. analyzer (each NMT 0.5%; total NMT 1.0%).

Absence of protein demonstrated by adsorbance at 650 nm (LOD 40 ppm)

OPTIMIZATION OF THE BIO-CATALYST BY PROTEIN ENGENNERING



1. IMMOBILIZED ENZYMES

Optimisation of the catalyst

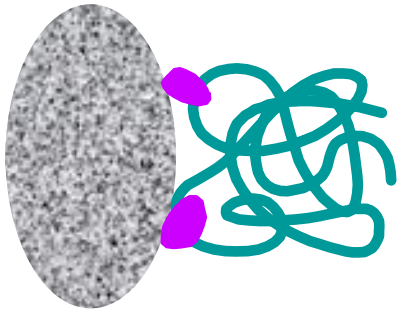
Design of the enzyme derivative

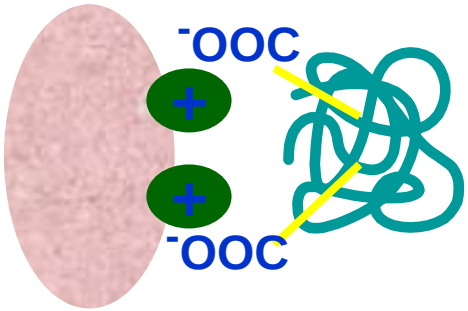
- Stability
- Re-use
- Catalytic properties

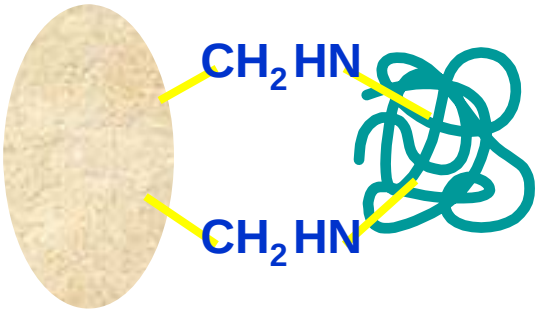
Avoid the Release of protein in the reaction medium

Relase of protein from the enzyme derivative

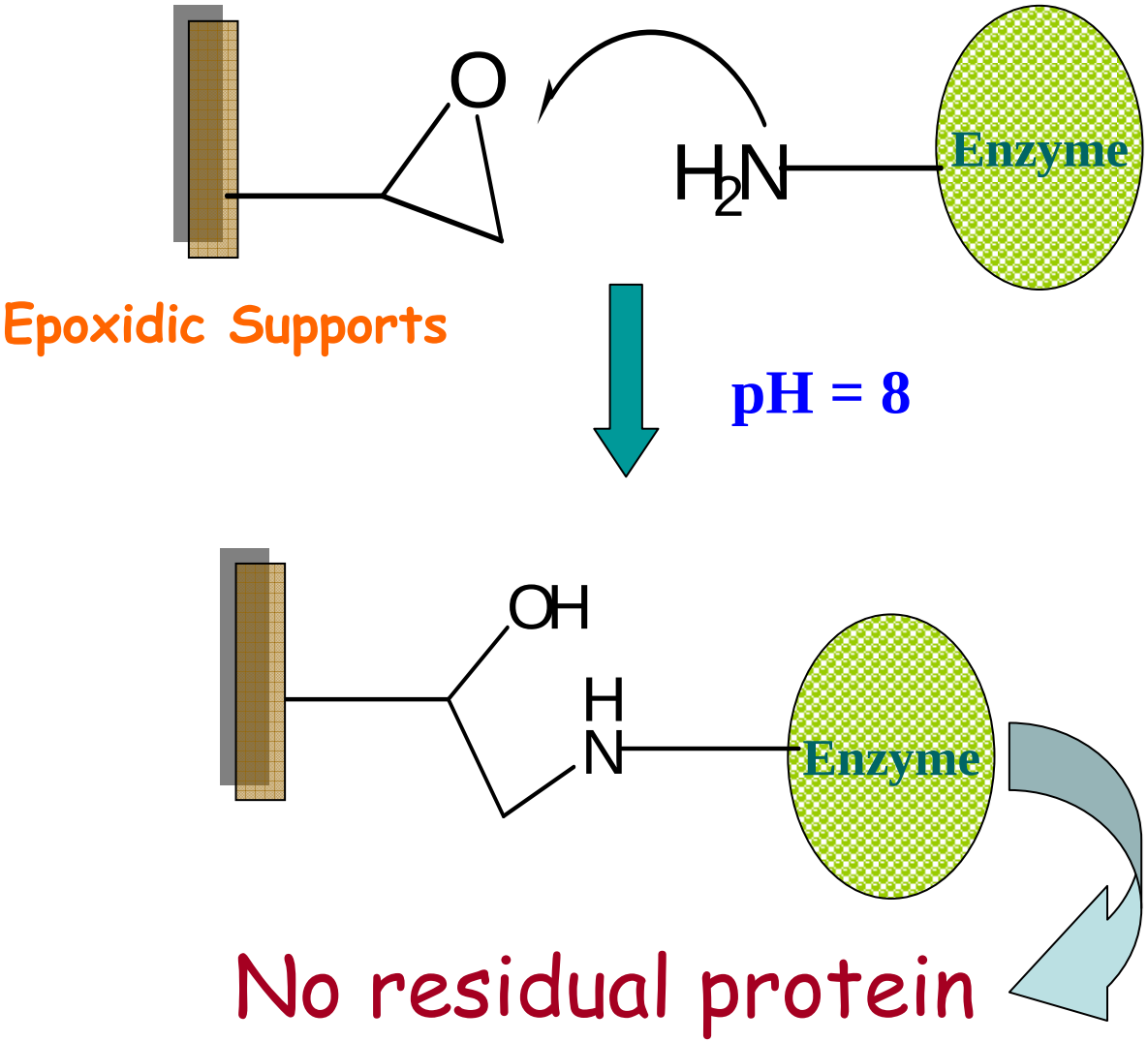
Extreme temperature and presence of co-solvents	HYDROPHOBIC ADSORPTION
Extreme temperature and pH; High Ionic strength	IONIC ADSORPTION
No release; Special case: multimeric enzymes	COVALENT ATTACHMENT



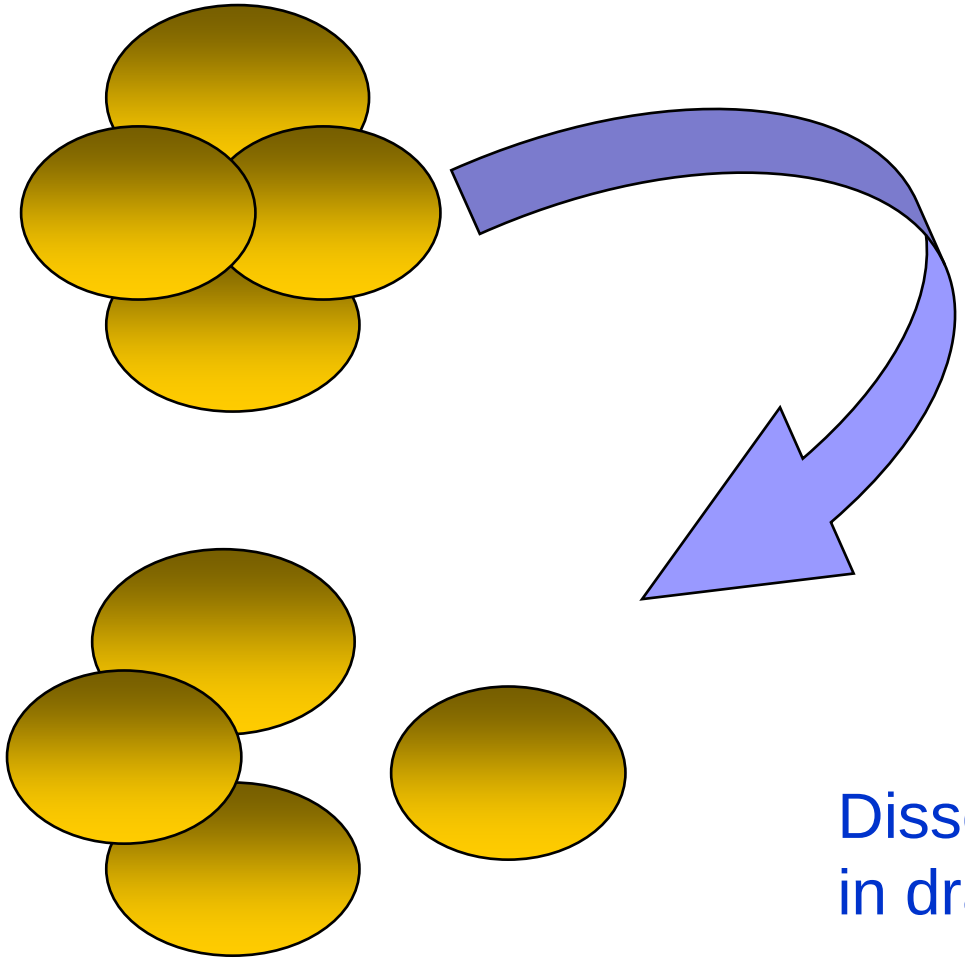




Covalent Immobilisation



Stabilization of Multimeric Enzymes

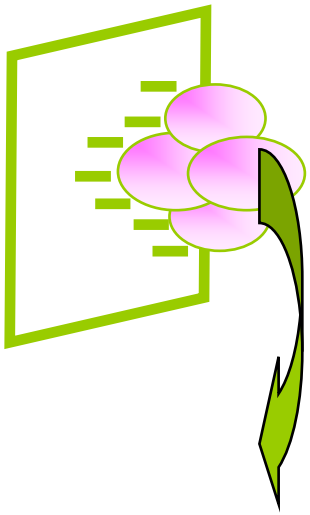


High pH
High temperature

Problem

Dissociation of the subunits
in drastic reaction conditions

Special case: Multimeric enzymes



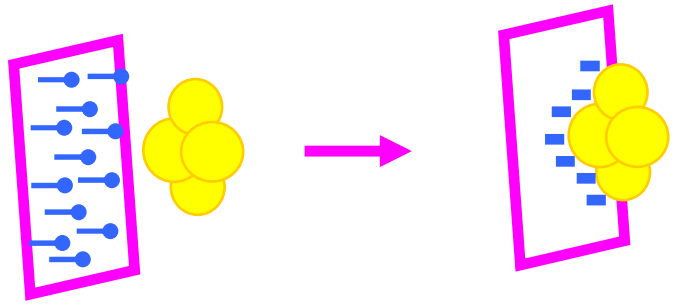
Covalent immobilisation

Dissociation of the subunits:
- **Low stability of the enzyme**
- **Residual protein in the final product**

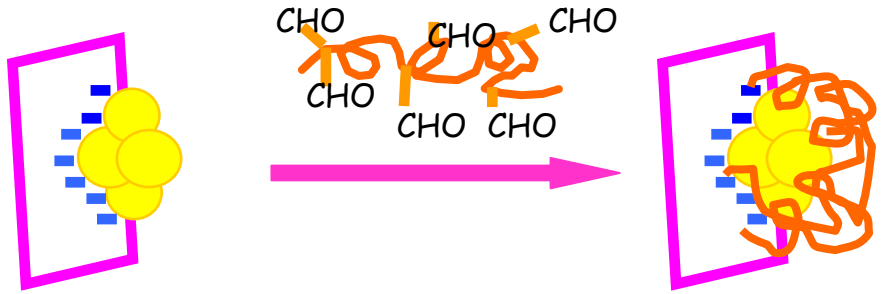
Knowledge of the catalyst engineering is essential

IMMOBILIZATION-STABILIZATION OF MULTIMERIC ENZYMES

1. immobilization

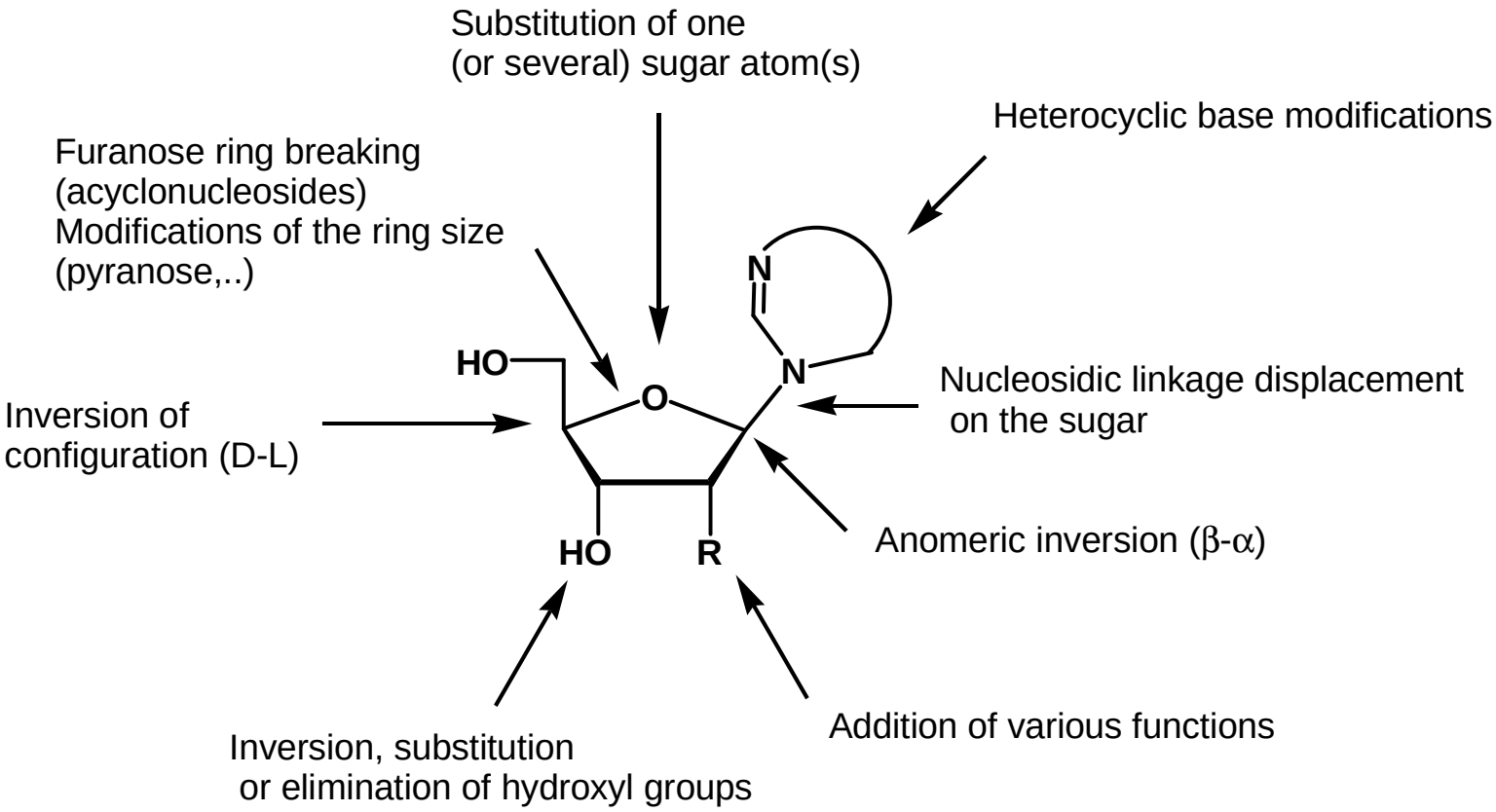


2. "*Subunit cross-linking*" with polifunctionalized molecules



NUCLEOSIDE ANALOGUES AND CHEMOTHERAPY

(=MODIFICATIONS OF THE BASE AND/OR SUGAR MOIETY)



SYNTHESIS OF THE *N*-GLYCOSYDIC BOND

STEREO-SELECTIVITY

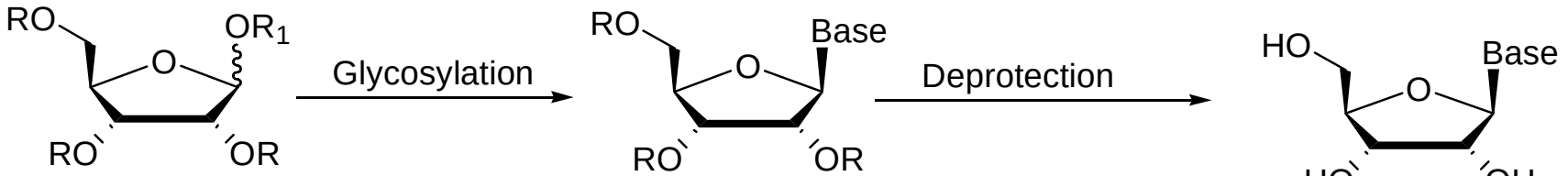
CHEMO-SELECTIVITY

REGIO-SELECTIVITY

N° Step → Impurities

CHEMICAL SYNTHESIS OF 2'-DEOXYNUCLEOSIDES

1. Synthesis of the ribonucleoside (glycosylation)

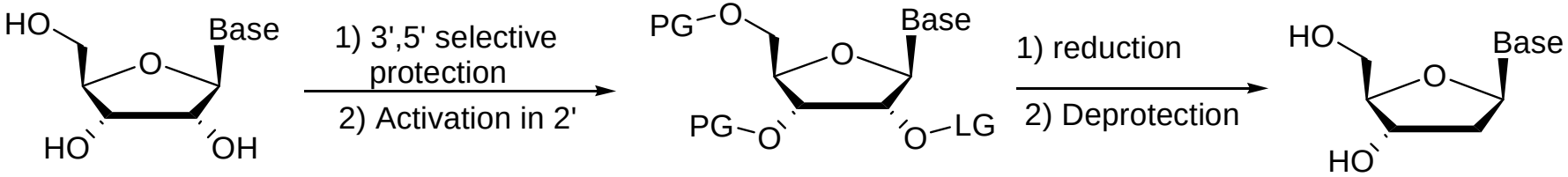


R=Bz, R_1=Ac

1

2

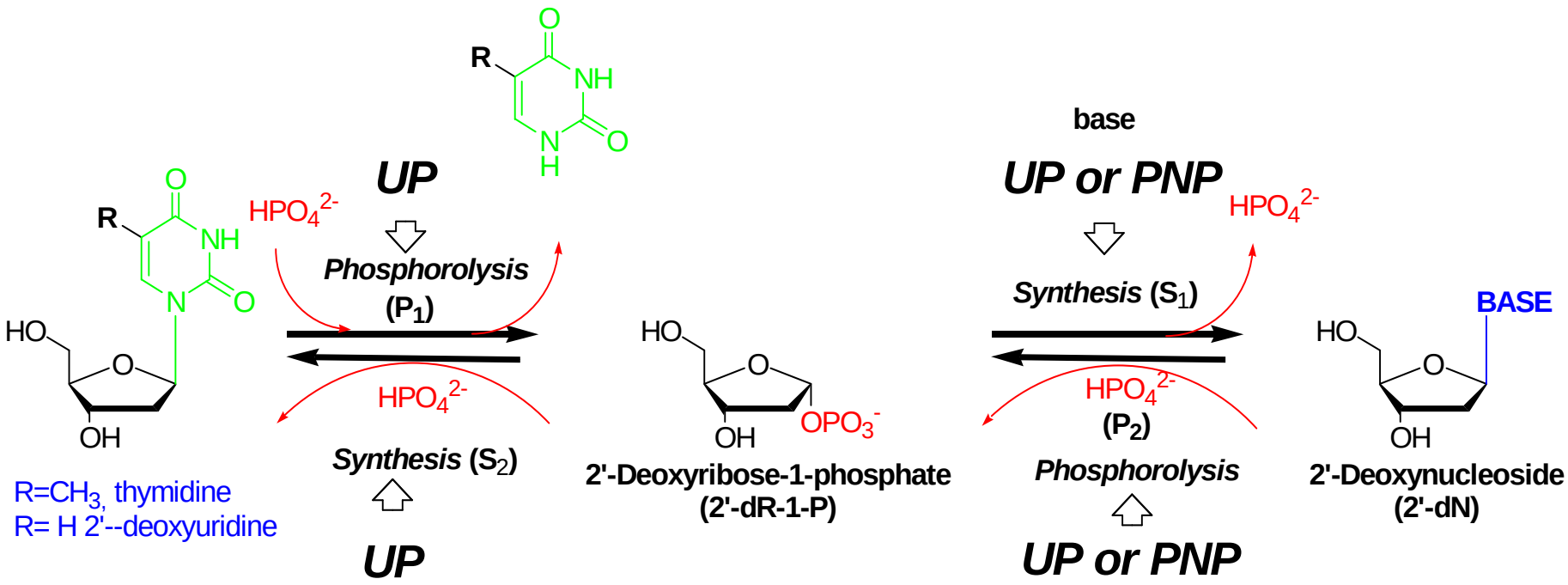
2. Selective radicalic reduction of the ribonucleoside in 2'



PG=*protecting group*
LG=*leaving group*

2 **3** **4**

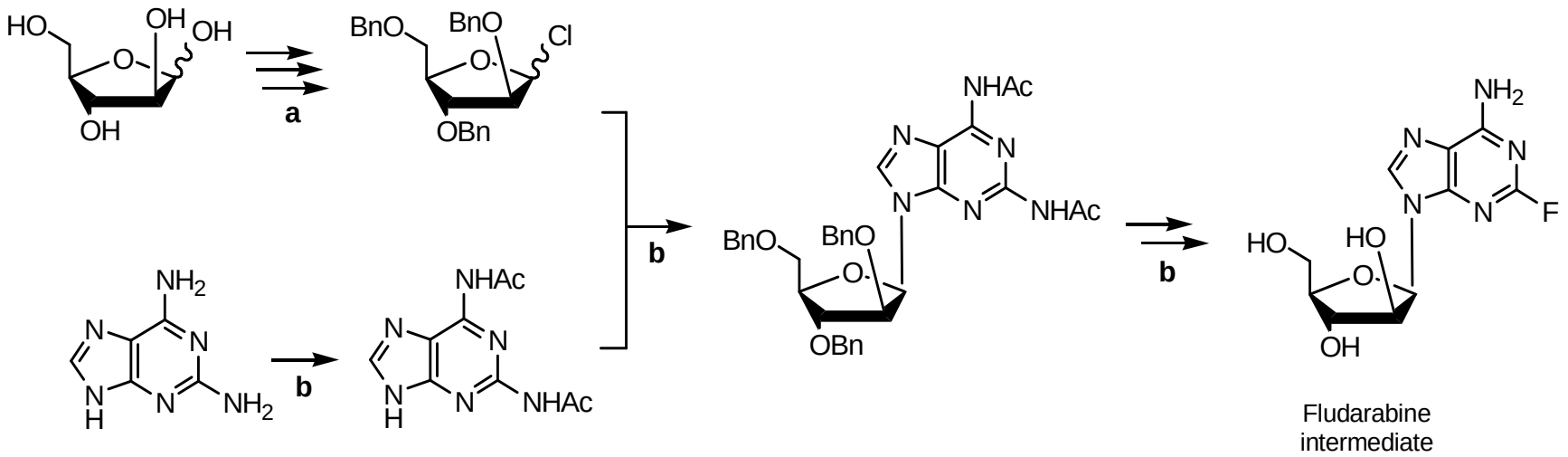
ONE-POT ENZYMATIC SYNTHESIS OF NUCLEOSIDES



Catalyzed by Phosphorilases

Enantioselective glycosilation: only beta-anomer

CHEMICAL SYNTHESIS OF FLUDARABINE



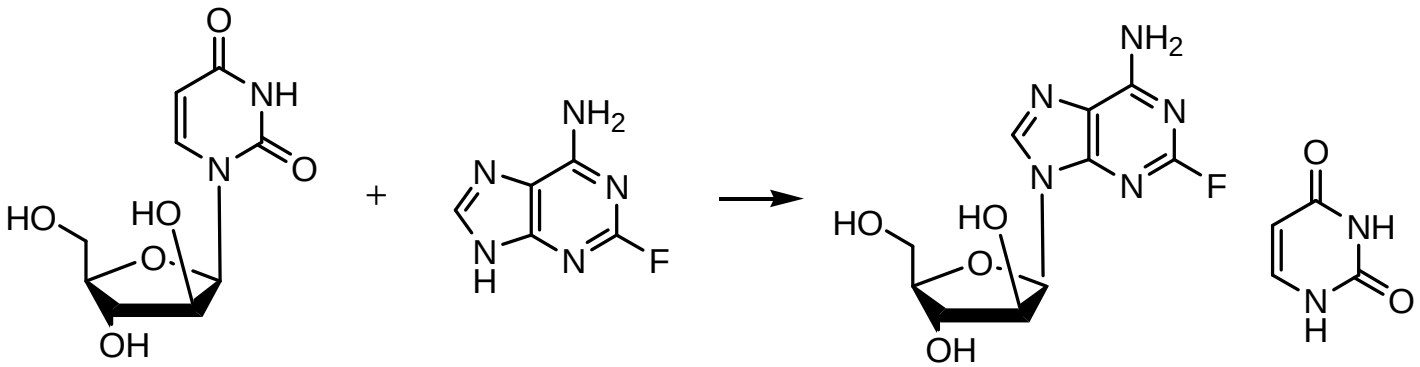
Fludarabine intermediate

a: Bioorganic & Medicinal Chemistry Letters, 15(3), 683-685; 2005
b: Huaxue Yanjiu Yu Yingyong, 18(5), 570-572; 2006

Chemical synthesis:
10 steps

ENZYMATIC SYNTHESIS OF FLUDARABINE

Enzymatic glycosilation



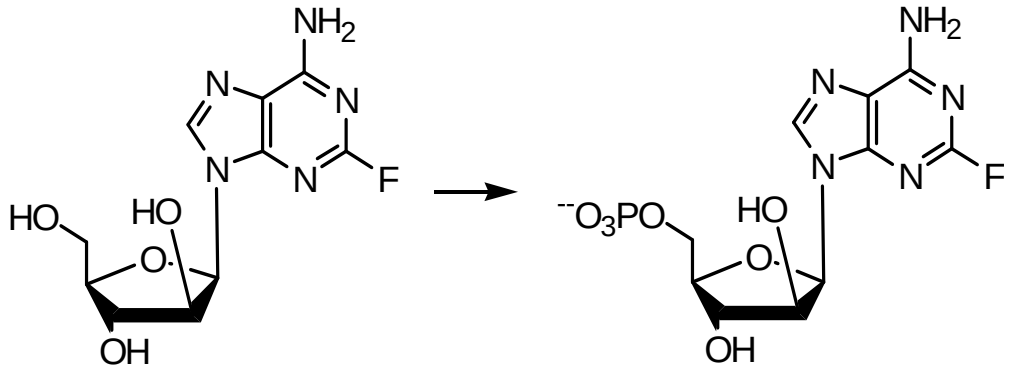
Fludarabine intermediate

Eur. Pat.Appl., 1835035, 19 Sep 2007
IP. com Journal, 6(10A), 9; 2006

Enzymatic reaction:
1 step
Cell paste
Water as solvent

Synthesis of Fludarabine monophosphate

PHOSPHORYLATION OF FLUDARABINE INTERMEDIATE



Fludarabine
5'-monophosphate

Chemical reaction
Triethylphospate as solvent

PCT Int. Appl., 2005040183, 06 May **2005**
Bulletin of the Chemical Society of Japan, **1969**, *42*, 3505
Chem. Pharm. Bull., **1995**, *43*, 210

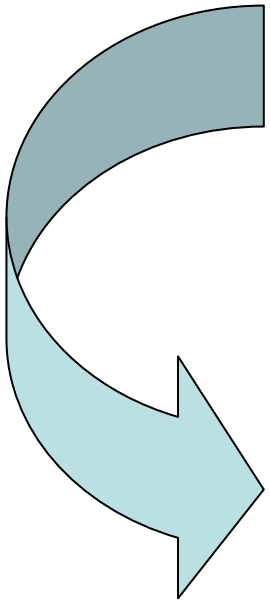
Purification of Fludarabine monophosphate

Chemo-enzymatic process:

Ultra filtration
Crystallization and wasching from water

Chemical process:

Crystallization from water



Control of quality:

Wool cells are used

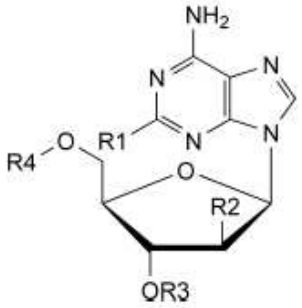
Residual from Microorganis:

Protein eliminated by ultra-filtration
Last reaction in organic solvent

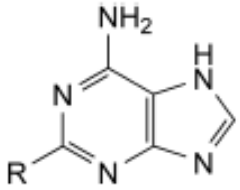
Nucleic acid controlled in API by
Eur.Ph 01/2005:0784
hybridization analysis in recombinant DNA

FLUDARABINE PHOSPHATE

Fludarabini phosphas



A. R1 = R2 = OH, R3 = H, R4 = PO_3H_2: 6-amino-9-(5-*O*-phosphono-β-D-arabinofuranosyl)-9*H*-purin-2-ol,



B. R = OH: 6-amino-7*H*-purin-2-ol,

D. R = F: 2-fluoro-7*H*-purin-6-amine,

C. R1 = F, R2 = OH, R3 = R4 = PO_3H_2: 9-(3,5-di-*O*-phosphono-β-D-arabinofuranosyl)-2-fluoro-9*H*-purin-6-amine,

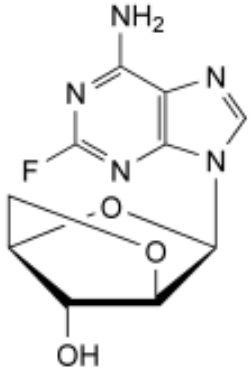
E. R1 = F, R2 = OH, R3 = R4 = H: 9-β-D-arabinofuranosyl-2-fluoro-9*H*-purin-6-amine,

F. R1 = O-C_2H_5, R2 = OH, R3 = H, R4 = PO_3H_2: 2-ethoxy-9-(5-*O*-phosphono-β-D-arabinofuranosyl)-9*H*-purin-6-amine,

G. R1 = F, R2 = Cl, R3 = H, R4 = PO_3H_2: 9-(2-chloro-2-deoxy-5-*O*-phosphono-β-D-arabinofuranosyl)-2-fluoro-9*H*-purin-6-amine,

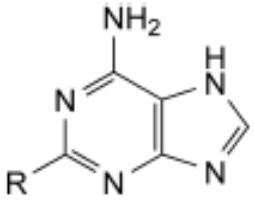
I. R1 = NH_2, R2 = OH, R3 = H, R4 = PO_3H_2: 9-(5-*O*-phosphono-β-D-arabinofuranosyl)-9*H*-purine-2,6-diamine,

J. R1 = OCH_3, R2 = OH, R3 = H, R4 = PO_3H_2: 2-methoxy-9-(5-*O*-phosphono-β-D-arabinofuranosyl)-9*H*-purin-6-amine,



H. 9-(2,5-anhydro-β-D-arabinofuranosyl)-2-fluoro-9*H*-purin-6-amine.

Potential Impurities obtained in the synthesis of fludarabine



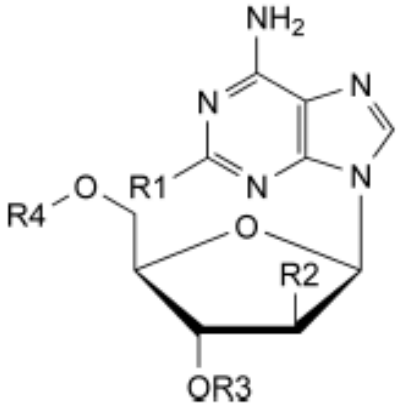
B. R = OH: 6-amino-7*H*-purin-2-ol,

Chemical: Unreacted impurity of diaminopurine
Enzymatic: Unreacted impurity of fluoroadenine

D. R = F: 2-fluoro-7*H*-purin-6-amine,

Chemical: degradation or by- product
Enzymatic: Unreacted starting material (F-adenine)

Potential Impurities obtained in the synthesis of Fludarabine Mono-Phosphate



A. R1 = R2 = OH, R3 = H, R4 = PO_3H_2: 6-amino-9-(5-*O*-phosphono-β-D-arabinofuranosyl)-9*H*-purin-2-ol,

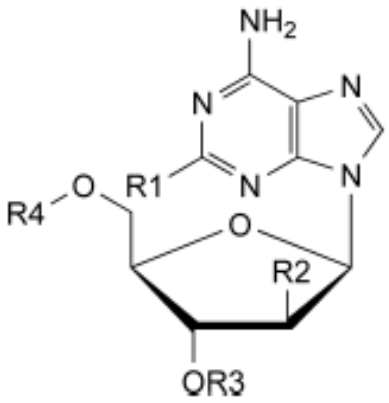
Chemical: from a by-product obtained during base modification
Enzymatic: from impurity B in F-Adenine

C. R1 = F, R2 = OH, R3 = R4 = PO_3H_2: 9-(3,5-di-*O*-phosphono-β-D-arabinofuranosyl)-2-fluoro-9*H*-purin-6-amine,

Chemical: by-product during phosphorylation
Enzymatic: byproduct during phosphorylation

E. R1 = F, R2 = OH, R3 = R4 = H: 9-β-D-arabinofuranosyl-2-fluoro-9*H*-purin-6-amine,

Chemical: unreacted Fludarabine int.
Enzymatic: unreacted Fludarabine int.



F. R1 = O-C_2H_5, R2 = OH, R3 = H, R4 = PO_3H_2: 2-ethoxy-9-(5-*O*-phosphono-β-D-arabinofuranosyl)-9*H*-purin-6-amine,

Chemical: from a by-product obtained during base modification

I. R1 = NH_2, R2 = OH, R3 = H, R4 = PO_3H_2: 9-(5-*O*-phosphono-β-D-arabinofuranosyl)-9*H*-purine-2,6-diamine,

Chemical: from a by-products obtained during base modification

J. R1 = OCH_3, R2 = OH, R3 = H, R4 = PO_3H_2: 2-methoxy-9-(5-*O*-phosphono-β-D-arabinofuranosyl)-9*H*-purin-6-amine,

Chemical: from a by-products obtained during base modification

Quality: Related Substances

Impurities	**Ph. Eur. name**	**Ph Eur limits**	**Chemo-Enzymatic**	**Chemical**
Fludarabine phosphate, 2-hydroxy analog	Impurity A	≤ 0.8%	0 - 0.2%	0.2-05%
Isoguanine	Impurity B	≤ 0.2%	ND	ND
Fludarabine diphosphate	Impurity C	≤ 0.4%	ND	0.2-0.3%
2-Fluoroadenine	Impurity D	≤ 0.1%	ND	ND
Fludarabine	Impurity E	≤ 0.2%	ND	ND
2-Ethoxyphosphate analog	Impurity F	≤ 0.2%		0.1%
Any other impurity	Impurity I Impurity J	≤ 0.1%	0 - 0.1%	0.1%
Total other impurities		≤ 0.5%	0 - 0.1%	0.2-0.3%
Total impurities		**≤ 2.0%**	**0 - 0.3%**	**0.9-1.2%**

Solvent used

Chemo-Enzymatic process
Water
Triethylphosphate
4 class II solvents and 2 class III solvents
Chemical process
Water
Triethylphosphate
7 class II solvents and 5 Class III solvents

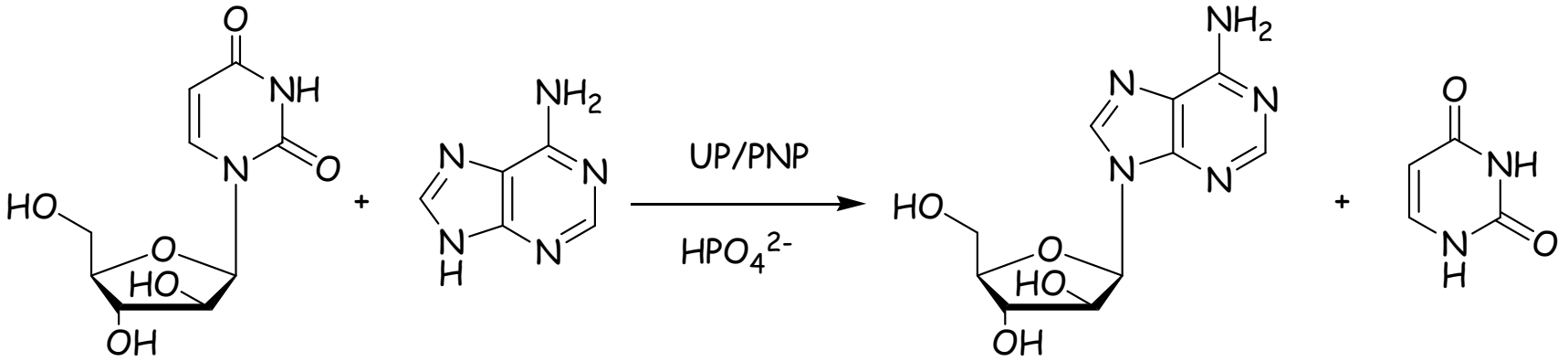
Other possible impurities

Chemo-Enzymatic process
Ara-U and Uracile
Chemical process
All intermediates
Pd used in the deprotection step
Alfa-anomer of fludarabine

Further Improvement:

1) Enzymatic Transglycosylation

Using Isolated And Immobilized Enzymes



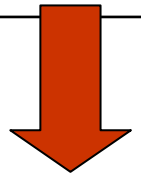
arabinosyluracil (ara-U) + adenine (ade) → arabinosyladenine (ara-A) + uracil (u)

Immobylized Enzymes

- UP: Pyrimidine phosphorylase from *Clostridium perfringens*
- DeoD2: Purine phosphorylase from *Aeromonas hydrophila*

Enzymatic synthesis of arabinosyladenine

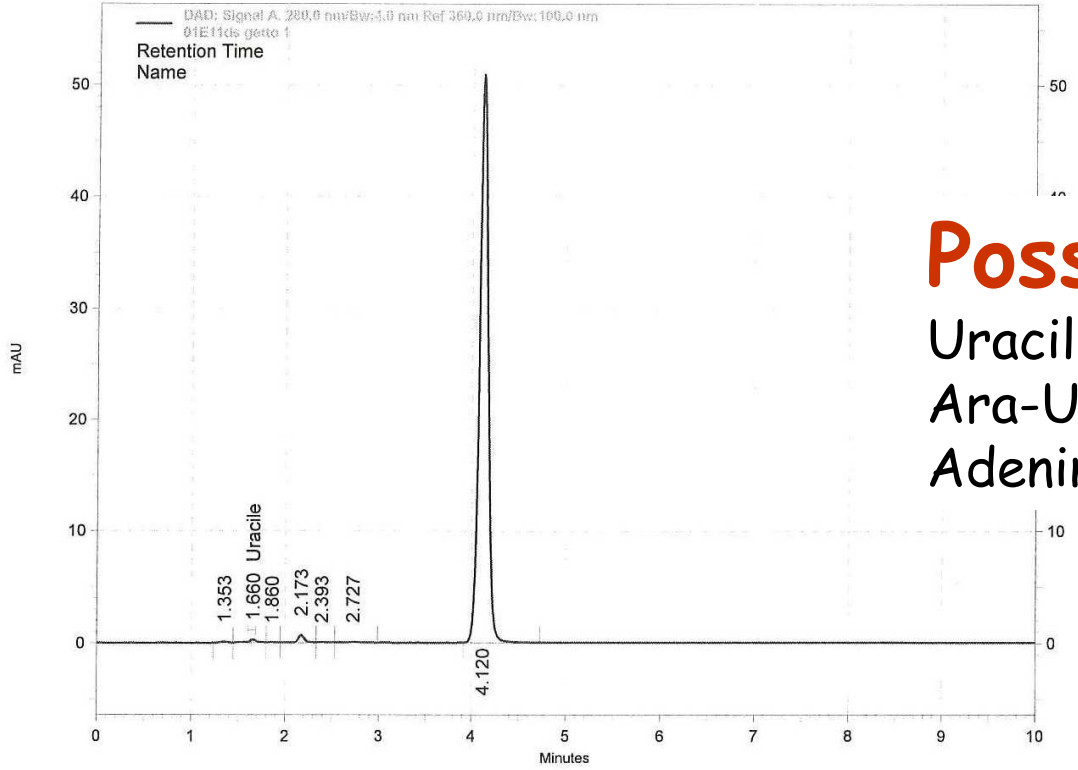
AraU (mM)	Ade (mM)	Volume (mL)	Cp UP (U)	Ah DeoD2 (U)	Conversion % (h)	Isolated yield (%)	Purity (%)
40	20	20	48	60	75 (48)	-	-
50	25	20	48	60	73 (48)	-	-
50	25	100	250	300	77 (26)	45	96
50	25	500	1250	1500	77 (26)	56	96
50	**25**	**2000**	**5000**	**6000**	**80 (26)**	**55**	**98**



Purification

Precipitation from water/DMF solution (reaction medium)
Filtration
Crystallyzation and Washing with water
Recovery: 55%

Product



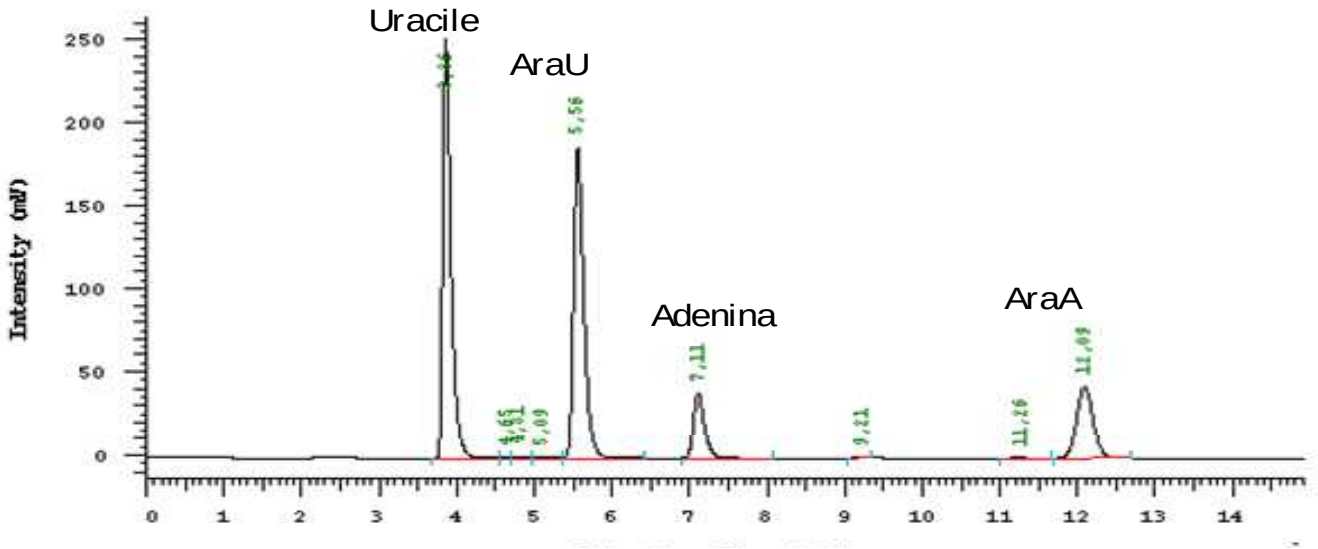
Possible impurities

Uracil	0,62%
Ara-U	1,05
Adenine	ND

DAD: Signal A, 280.0 nm/Bw:4.0 nm Ref 360.0 nm/Bw:100.0 nm Results

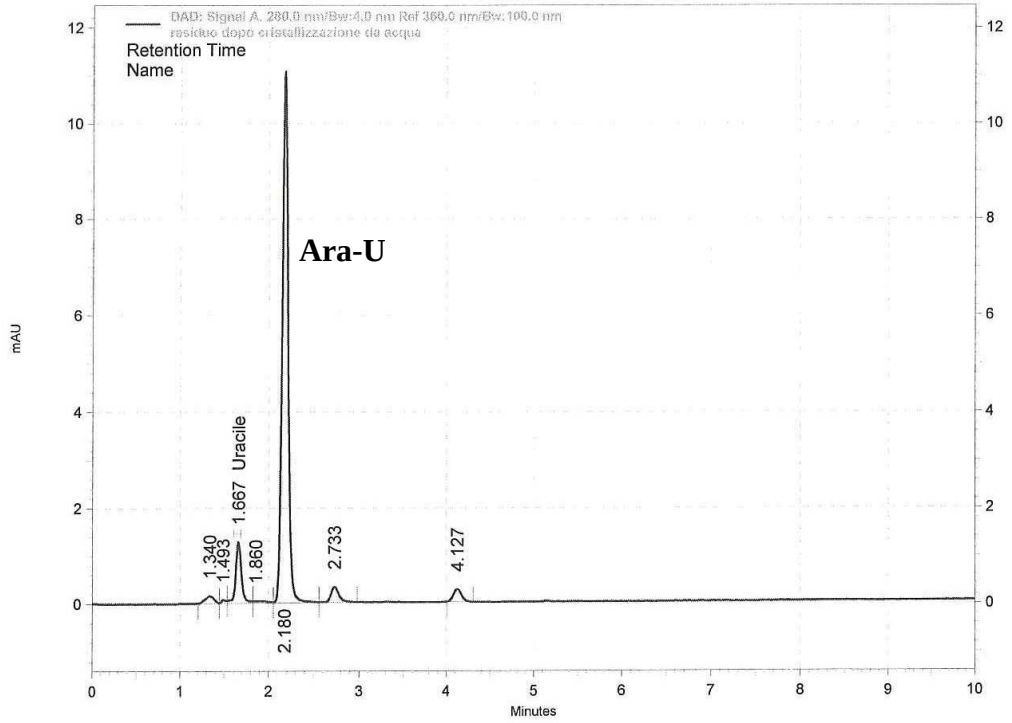
Retention Time	Area	Area %	Height	Height %	Name
1.353	79233	0.18	13975	0.20	
1.660	279533	0.62	39827	0.57	Uracile
1.860	45055	0.10	6153	0.09	
2.173	471242	1.05	88604	1.27	
2.393	48358	0.11	6924	0.10	
2.727	79229	0.18	8955	0.13	
4.120	43869546	97.77	6825315	97.65	

Mother liquor



No.	RT	Area	Conc 1	BC	Height	Area %
1	3,86	1819948	39,861	BV	251964	39,861
2	4,65	1109	0,024	VV	155	0,024
3	4,81	8459	0,185	VV	1023	0,185
4	5,09	6148	0,135	VV	451	0,135
5	5,56	1696461	37,156	VB	186851	37,156
6	7,11	383602	8,402	BB	38922	8,402
7	9,21	1477	0,032	BB	148	0,032
8	11,26	2574	0,056	BB	177	0,056
9	12,09	645994	14,149	BB	43043	14,149
		4565772	100,000		522734	100,000

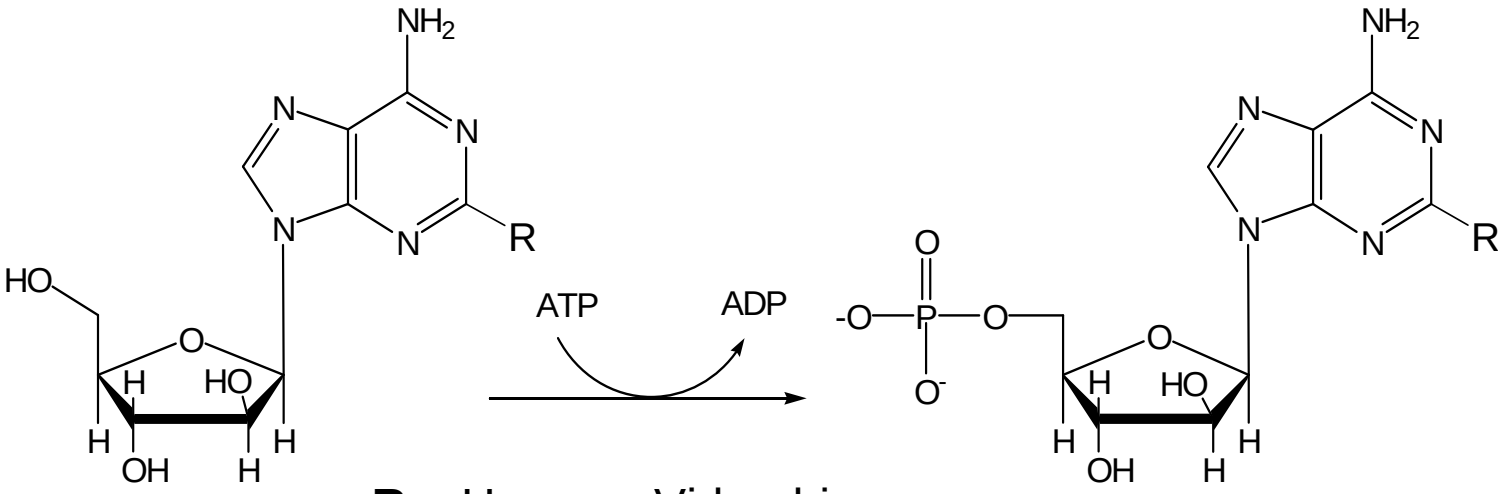
Ara-U can be recovered and reused



DAD: Signal A, 280.0 nm/Bw:4.0 nm Ref 360.0 nm/Bw:100.0 nm Results

Retention Time	Area	Area %	Height	Height %	Name
1.340	139352	1.69	19851	1.13	
1.493	30514	0.37	8688	0.49	
1.667	767467	9.29	170203	9.65	Uracile
1.860	51796	0.63	4591	0.26	
2.180	6787864	82.21	1483022	84.10	
2.733	261517	3.17	42454	2.41	
4.127	218336	2.64	34592	1.96	

Further Improvement:
2) Enzymatic phosphorylation catalysed by immobilized Kinases



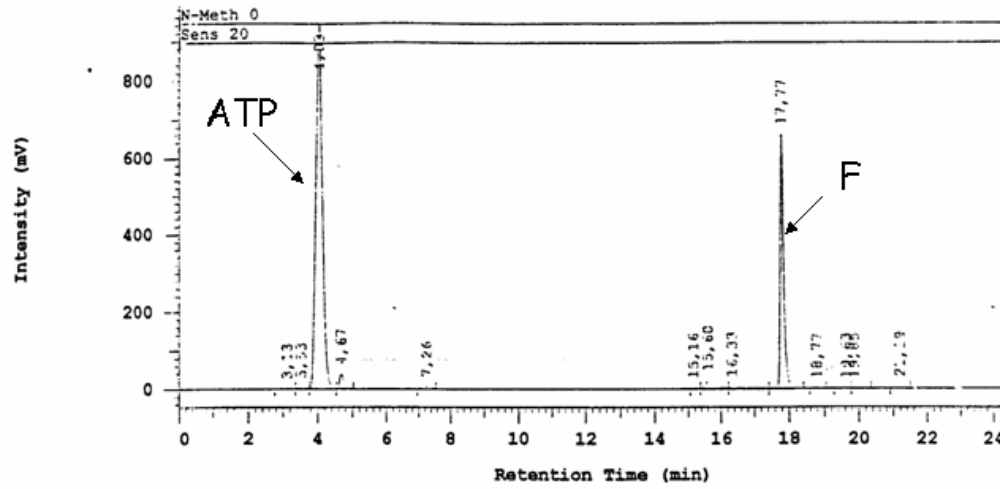
R = H ⟶ Vidarabine

R = F ⟶ Fludarabine

Immobylized Enzyme

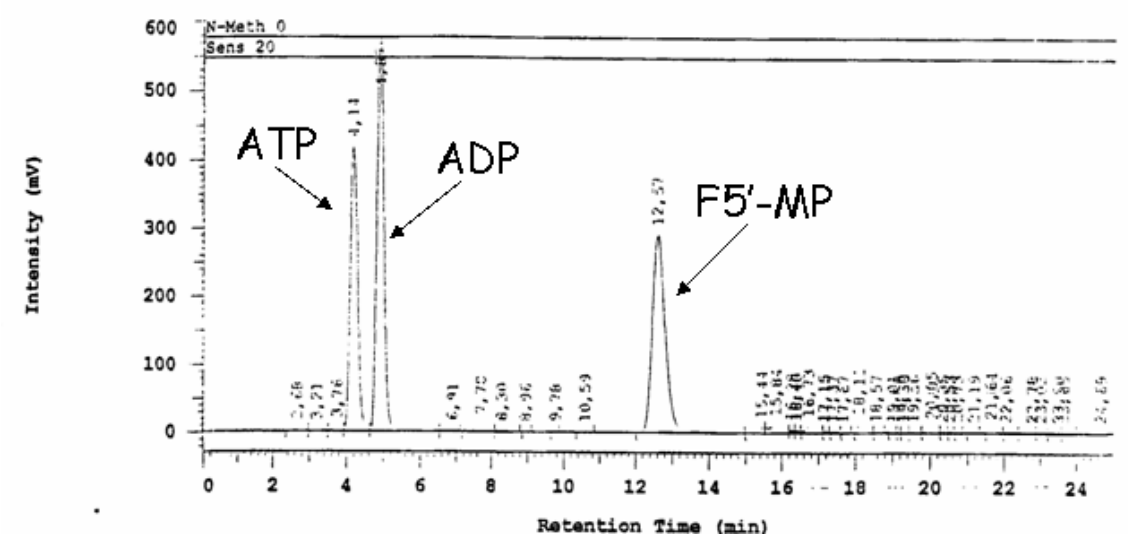
DADA: Reconbinant Kynase from *Dictyostelium discoideum*

Fludarabine (R=F)



t=0

t=19 h



Conversion 96% (18 g/L)