UNIVERSITY OF STUDIES OF MILAN-BICOCCA

Federico Lami

Design and synthesis of a new Toll Like Receptor 4 agonist-based Antibody Drug Conjugate for cancer immunotherapy.

PATTERN RECOGNITION RECEPTORS: TOLL-LIKE RECEPTOR 4

PATTERN RECOGNITION RECEPTORS

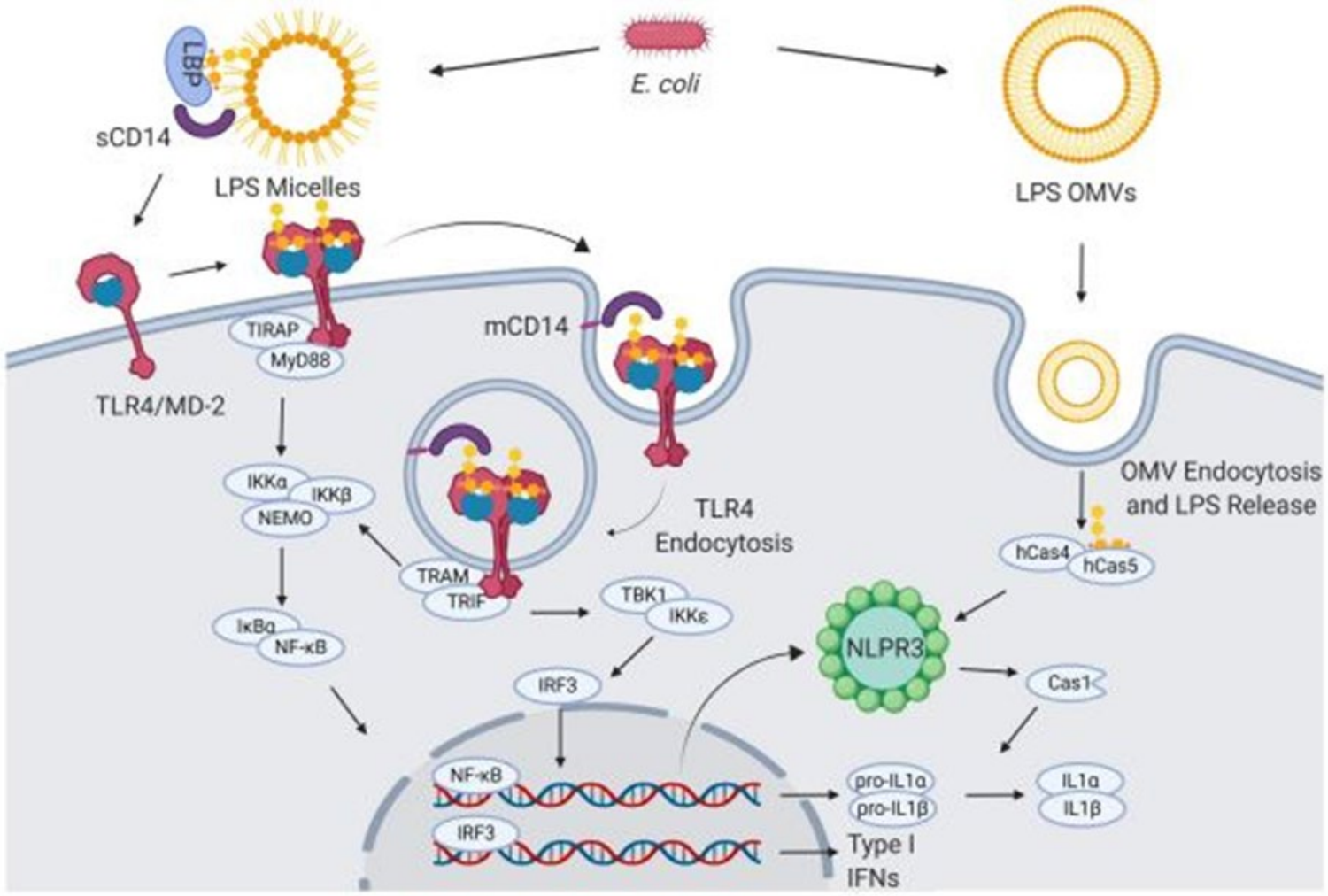
- Present on Innate Immune System cells.
- Sensing of Pathogen or Damage Associated Molecular Patterns (PAMPs or DAMPs).

TOLL-LIKE RECEPTOR 4

- Sensing of Gram-negative bacteria.
- Lipopolysaccharide (LPS) is the natural ligand.
- Receptor is a TLR4/MD2 dimer.
- LPS binds to MD2 part of complex.

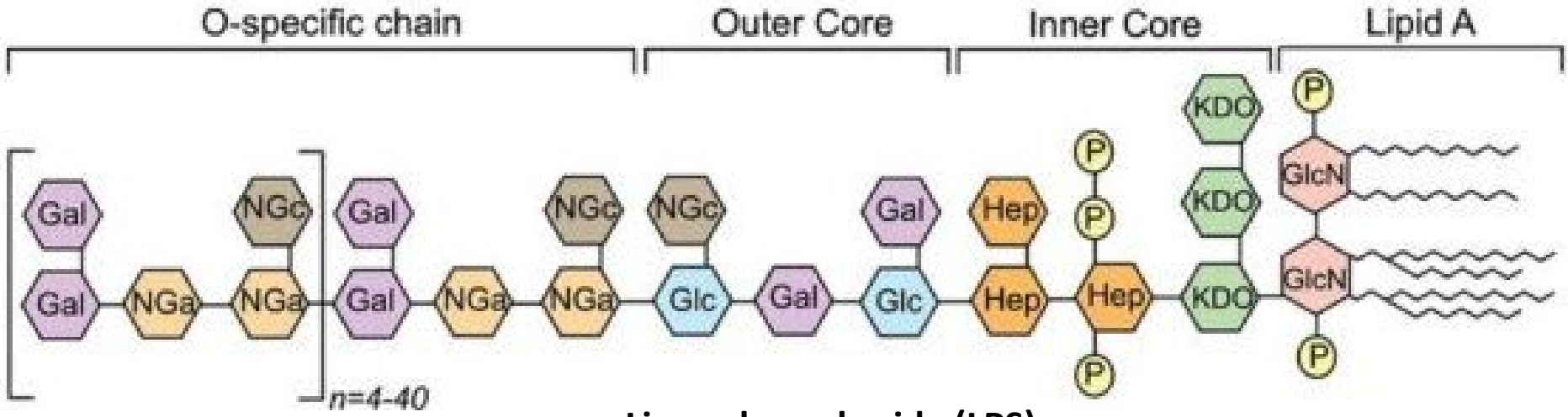
EXPOLITING TLR4 AGONISTS

- **Vaccine adjuvants**: new vaccines are more safe but less active
- Increase vaccine activity with adjuvants
- Cancer thrives in an environment of chronic inflammation.
- **Cancer immunotherapy**: awaken immune system towards cancer cells.



"Increasing the Chemical Variety ofSmall-Molecule-Based TLR4Modulators: An Overview", Romerio A. Peri F., Frontiers of Immunology *[2020], "***Clinical cancer immunotherapy: Current progress and prospects",** C. Liu, M. Yang, D. Zhang, M. Chen, D. Zhu**,** Frontiers in Immunology *[2022].*

LIPOPOLISACCHARIDE (LPS) STRUCTURE

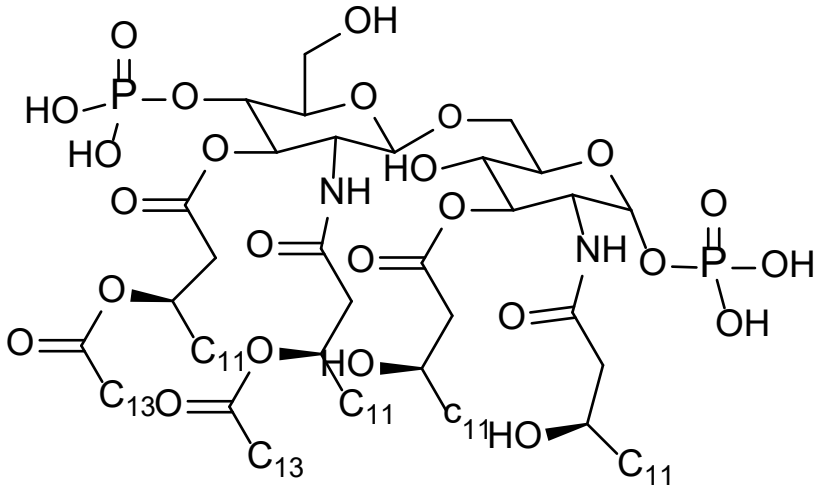


Lipopolysaccharide (LPS)

LPS

Hydrophilic Region:
- O-specific chain
- Outer Core
- Inner Core

Lipophilic region:
- Lipid A



Lipid A

LIPID A

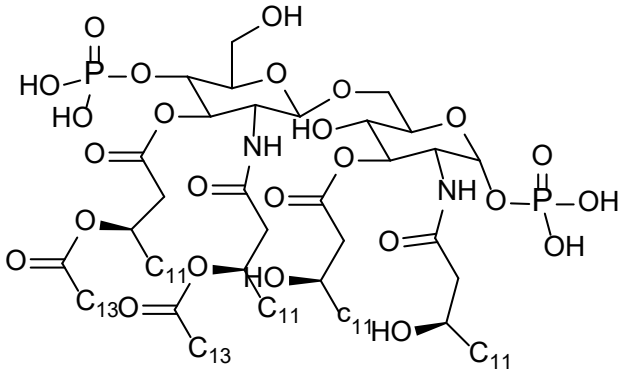
- Minimal immunogenic part of LPS

Disaccharide core:
- 2 Glucosamines
- 2 Phosphates

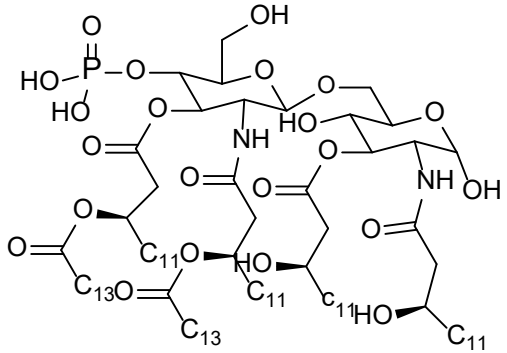
Lipidic tail:
- several acyl chains of different lenght and functionalization

"The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex.", Park B. S. *et al.*, *Nature* **458**, 1191–1195 *[2009]*.

SYNTHETIC TLR4 AGONISTS: LIPID A MIMETICS



Lipid A



MPLA

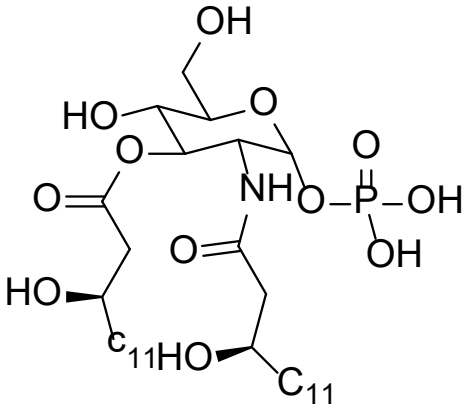
Monophosphoryl Lipid A

Extraction from natural sources:

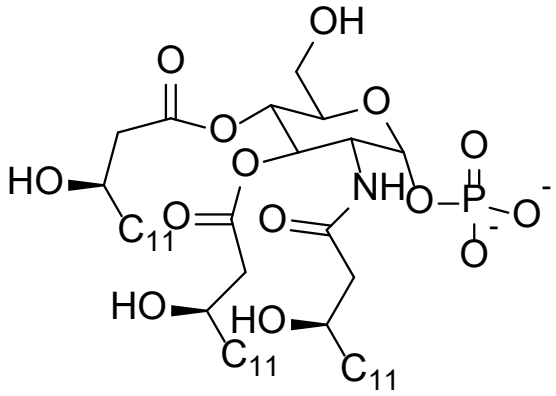
- Non homogeneous product

Synthetic approach:

- Use of many protective groups
- Long (24 steps) and Expensive (200 EUR/mg)



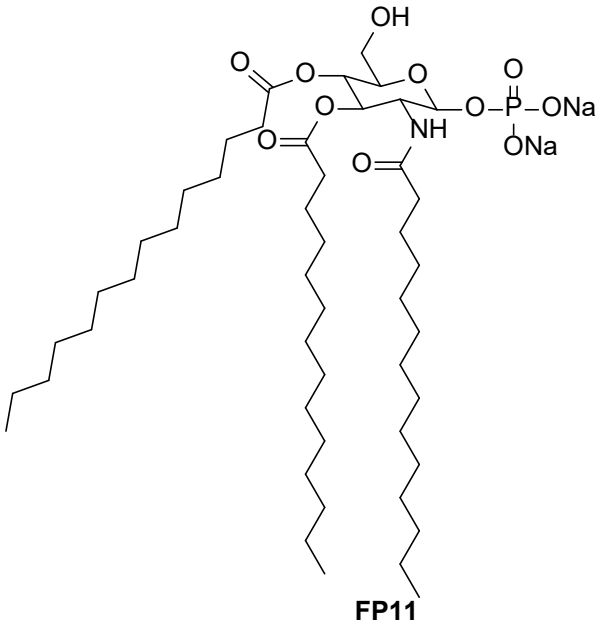
Lipid X



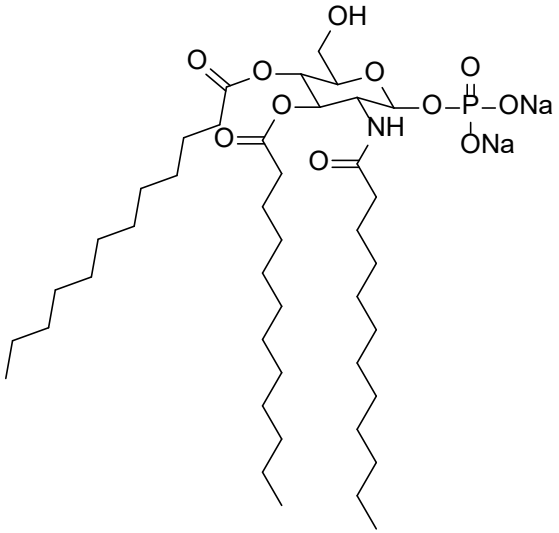
SDZ-MRL-953

"SDZ MRL 953, a novel immunostimulatory monosaccharidic lipid A analog with an improved therapeutic window in experimental sepsis",
C Lam, E Schütze, J Hildebrandt, H Aschauer, E Liehl, I Macher, P Stütz; *ANTIMICROBIAL AGENTS AND CHEMOTHERAPY*, p. 500-505, *[1991]*.

FIRST GENERATION OF AGONISTS: FP11/FP18



FP11



FP18

Journal of Medicinal Chemistry

pubs.acs.org/jmc

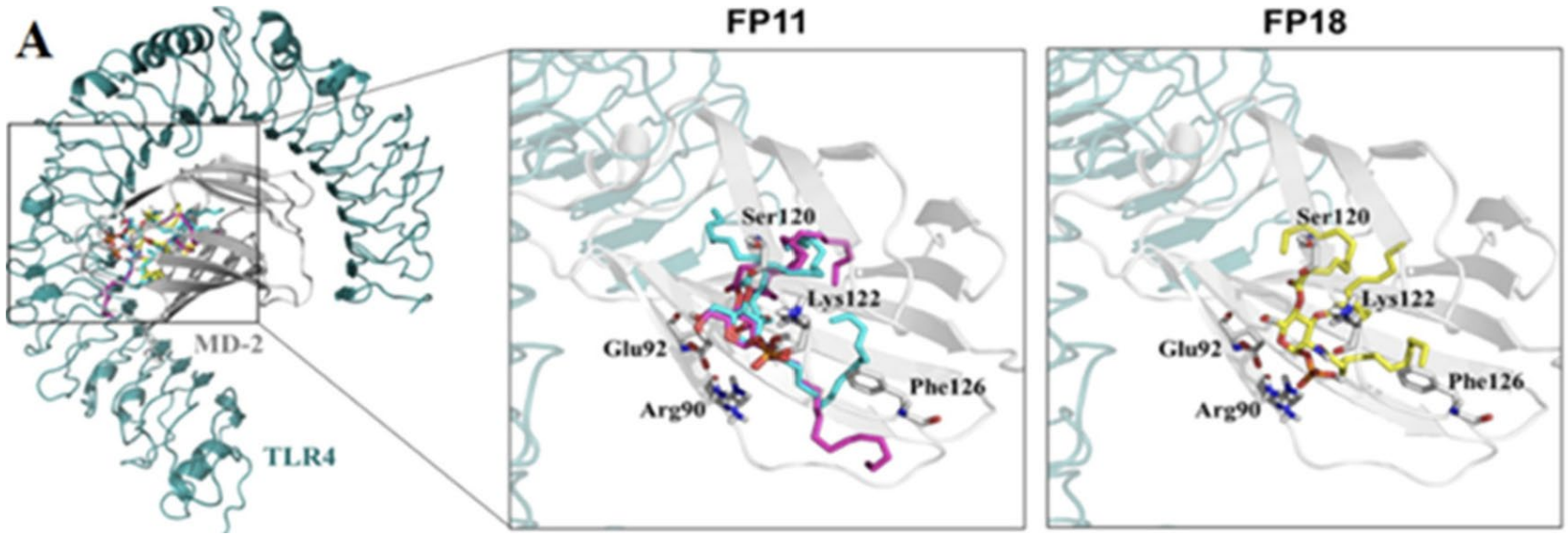
Article

Synthetic Glycolipids as Molecular Vaccine Adjuvants: Mechanism of Action in Human Cells and In Vivo Activity

Fabio A. Facchini, Alberto Minotti, Andrea Luraghi, Alessio Romerio, Nicole Gotri, Alejandra Matamoros-Recio, Andrea Iannucci, Charys Palmer, Guanbo Wang, Rebecca Ingram, Sonsoles Martin-Santamaria, Grisha Pirianov, Marco De Andrea, Miguel A. Valvano, and Francesco Peri*

Cite This: *J. Med. Chem.* 2021, 64, 12261–12272

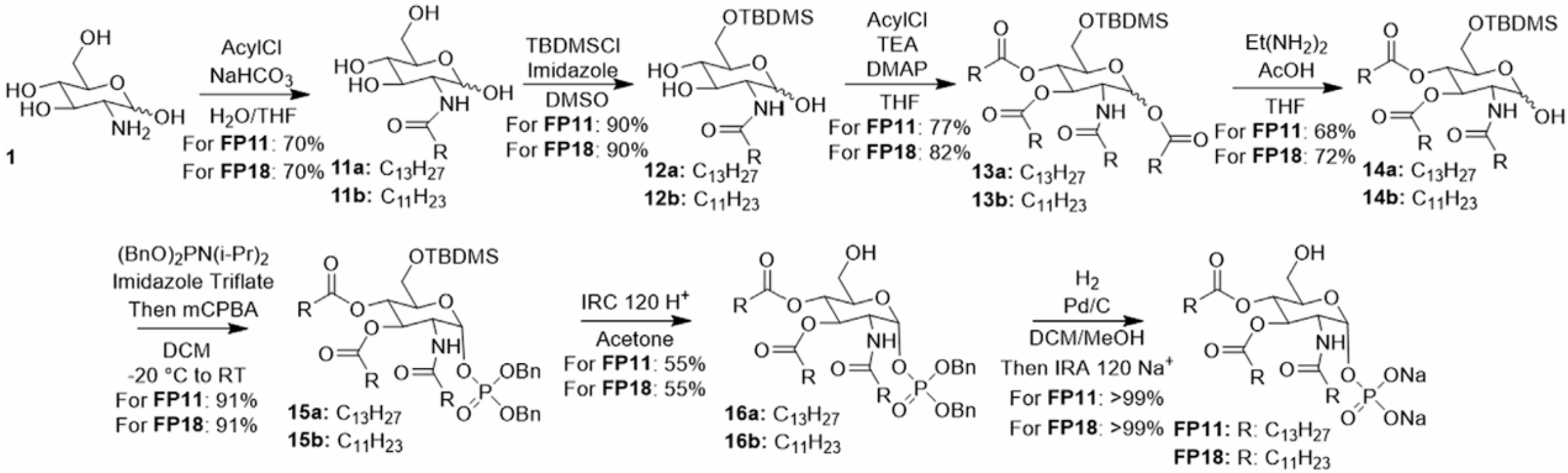
Read Online



BINDING SCORE
-3.8 kcal/mol

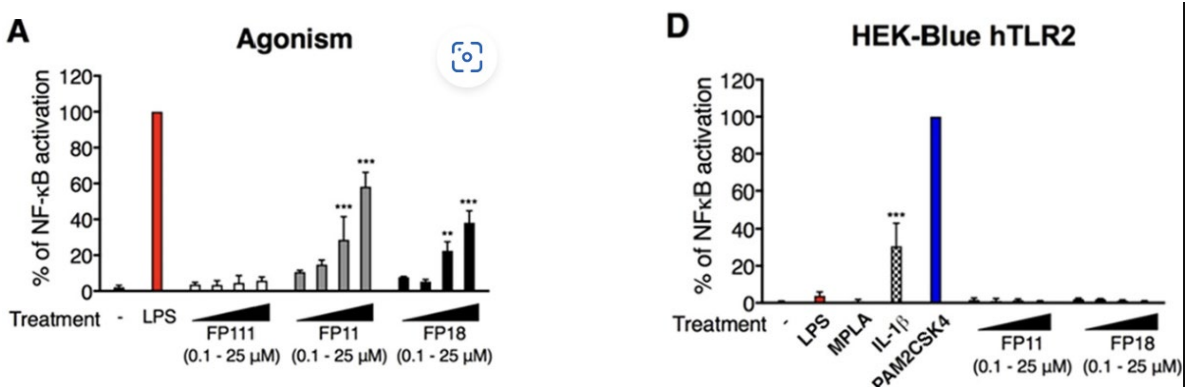
BINDING SCORE
-2.7 kcal/mol

FIRST GENERATION OF AGONISTS: SYNTHESIS

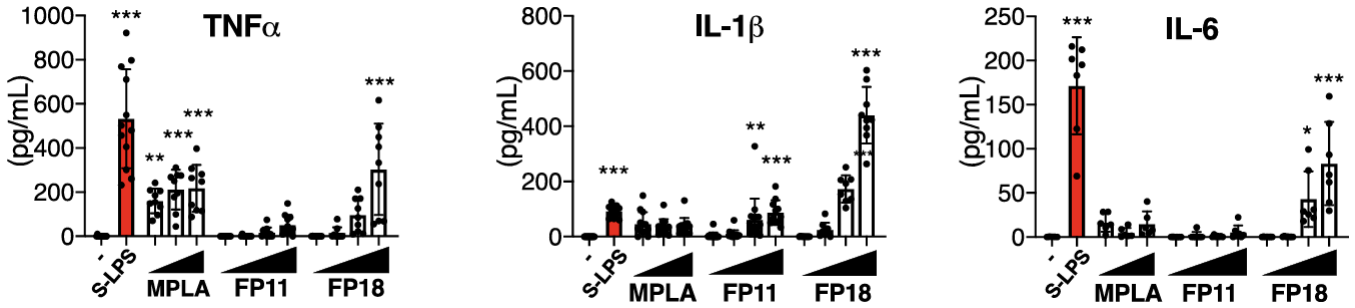


"Cleaner synthesis of preclinically validated vaccine adjuvants", A. Romerio, F. Peri, *Front. Chem., [2023]*

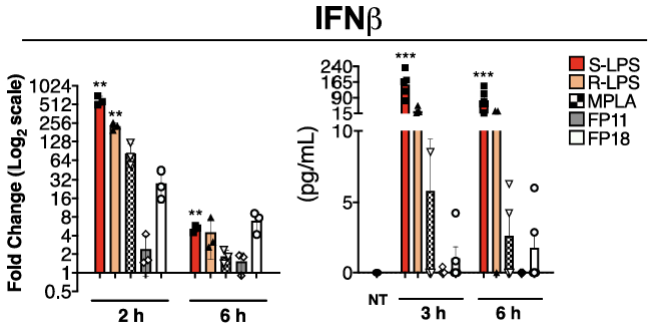
FIRST GENERATION OF AGONISTS: BIOLOGICAL ACTIVITY



In vitro nF-κB traslocation in Hek-Blue TLR4 and TLR2 cells



MyD-88 dependent cytokines production



TRAM/TRIF dependent IFNβ production

IN VIVO STUDIES ON OVOALBUMIN IMMUNIZED MICE

FP18 IS A BETTER ADJUVANT IN VIVO THAN FP11

TOWARDS A NEW GENERATION OF AGONISTS

International Journal of *Molecular Sciences*

MDPI

Review

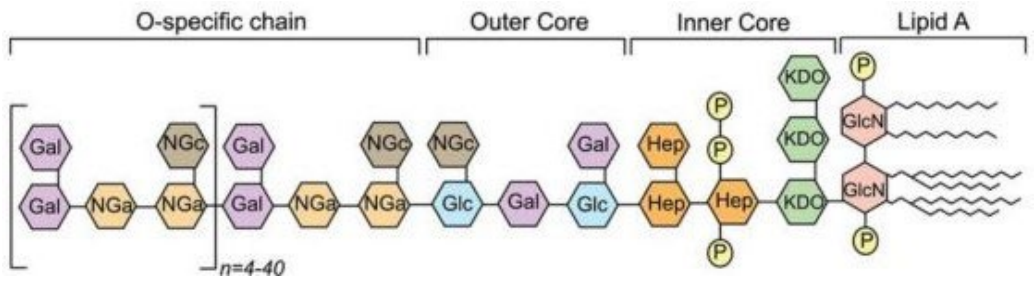
The Role of Carbohydrates in the Lipopolysaccharide (LPS)/Toll-Like Receptor 4 (TLR4) Signalling

Florent Cochet and Francesco Peri *

Department of Biotechnology and Biosciences, University of Milano Bicocca, Piazza della Scienza, 2, 20126 Milano, Italy; f.cochet@campus.unimib.it

* Correspondence: francesco.peri@unimib.it; Tel.: +39-02-6448-3453

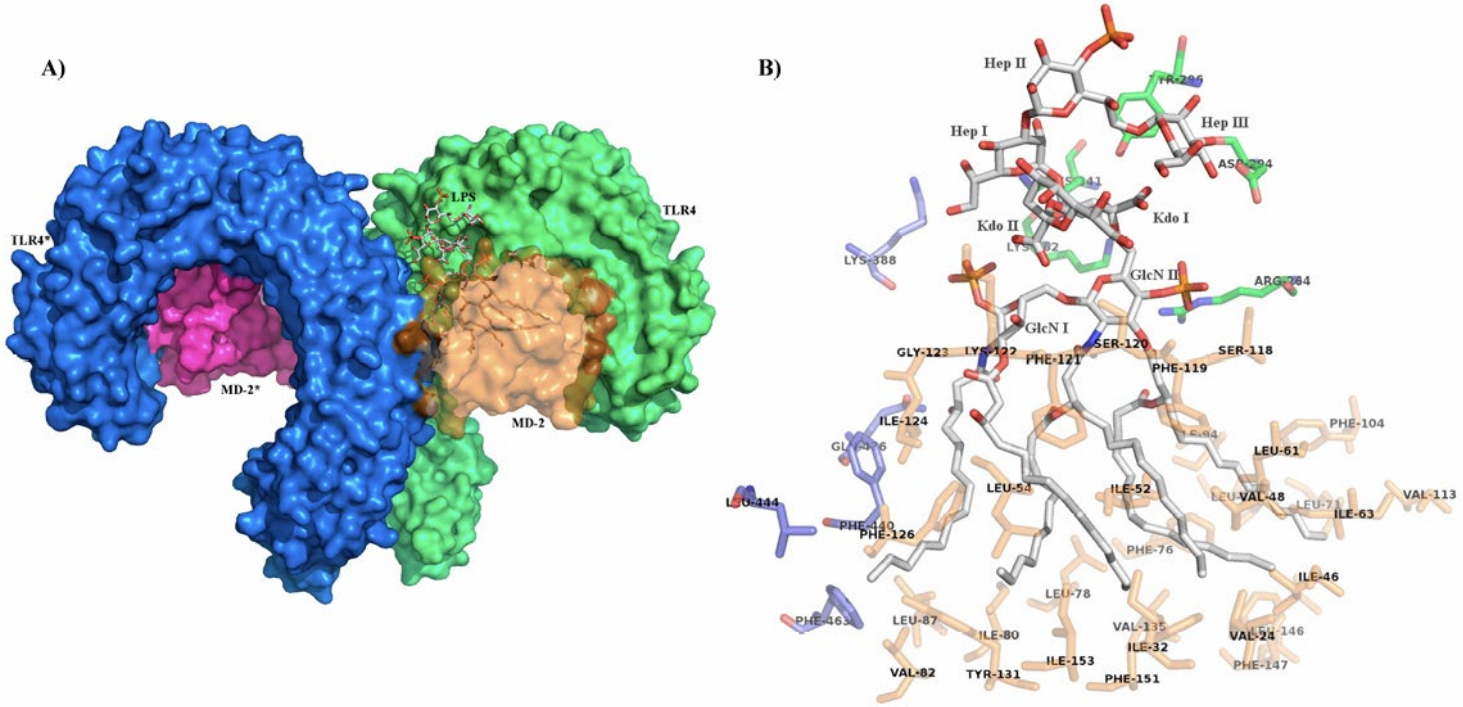
Received: 24 July 2017; Accepted: 30 October 2017; Published: 3 November 2017



INNER CORE KDO MOIETY INCREASES LIPID A ACTIVITY

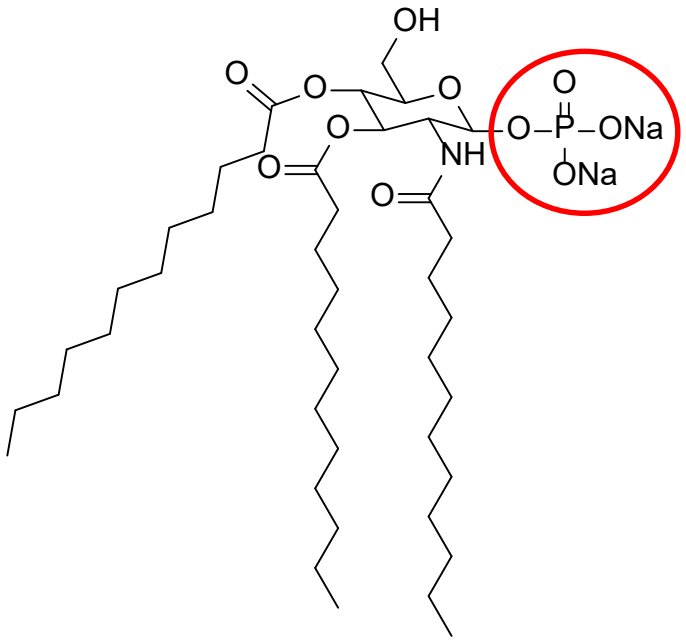
Kdo I, Hep I, and Hep III are interacting with the TLR4 residues Y296, K341, and D294, promoting MD2/TLR4 dimerization.

Fully synthetic lipid A containing Kdo units (also called Re-LPS) are always more active than their counterparts lacking Kdo.

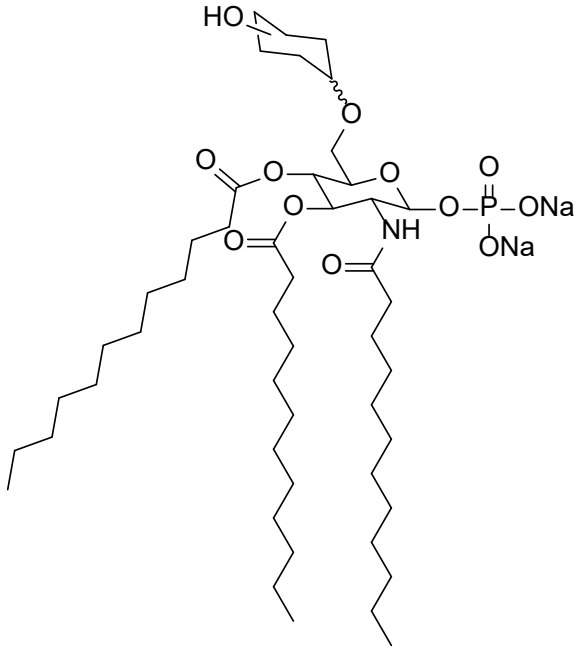


Interactions of Kdo and Hep moieties with TLR4

TOWARDS A NEW GENERATION OF AGONISTS



FP18



I

GLYCOSILATION ATTEMPTS RESULTED IN FAILURE DUE TO INSTABILITY OF THE PHOSPHATE GROUP IN ANOMERIC POSITION.

NEW GENERATION OF AGONISTS: FP20 AND GLYCO-FP20

ACS OMEGA

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Article

Overcoming Challenges in Chemical Glycosylation to Achieve Innovative Vaccine Adjuvants Possessing Enhanced TLR4 Activity

Alessio Romerio, Ana Rita Franco, Melanie Shadrick, Mohammed Monsoor Shaik, Valentina Artusa, Alice Italia, Federico Lami, Alexei V. Demchenko, and Francesco Peri*

Cite This: *ACS Omega* 2023, 8, 36412−36417

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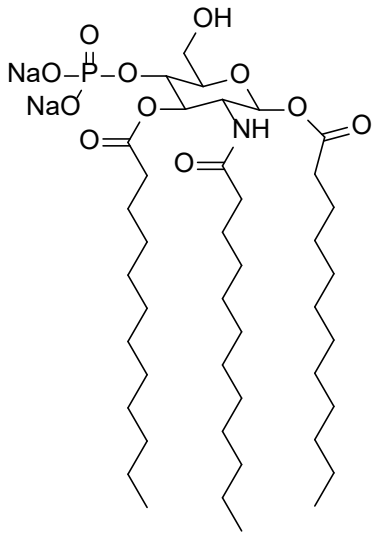
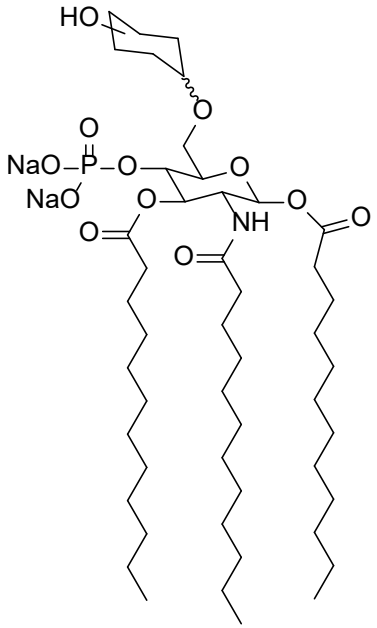
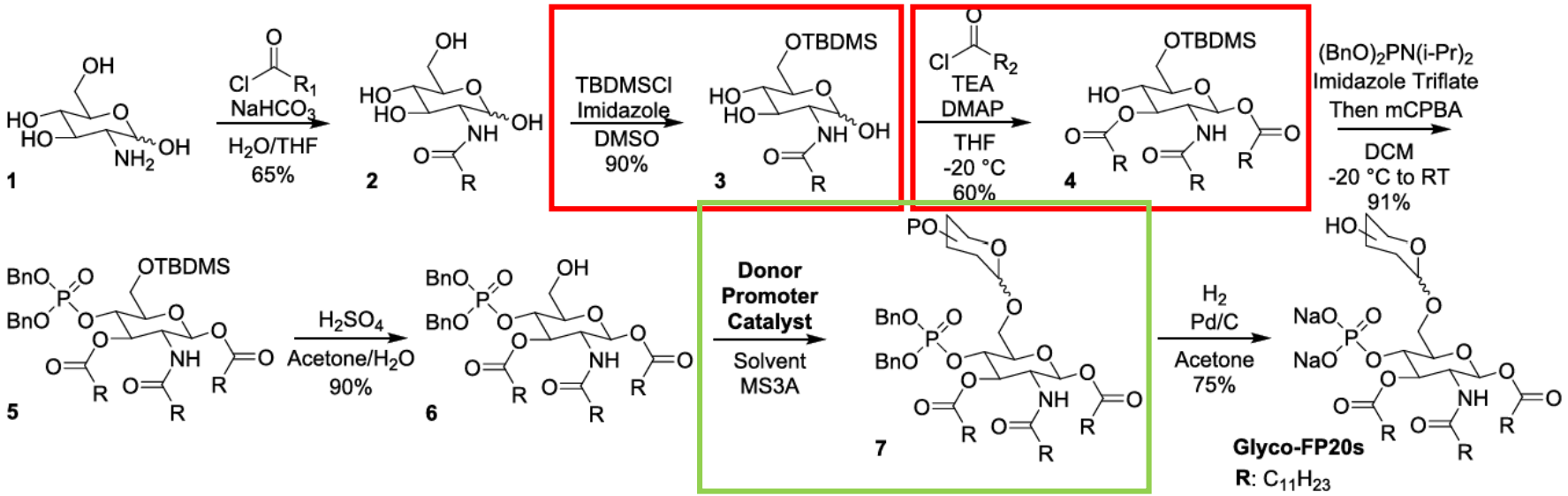


FP20



Glyco-FP20

NEW GENERATION OF AGONISTS: SYNTHESIS



Conditions Used in Early FP20 Glycosylation Attempts

Donors	Entry	Donor (Eq)	Solvent (M)	Promoter (Eq)	Catalyst (Eq)	Temperature (°C)	Yield (%)
a	1	a (3)	DCM (0.05)	N/A	TMSOTf (0.5)	0	N/D
	2	a (3)	DCM (0.05)	N/A	TMSOTf (0.5)	-20	N/D
	3	a (3)	DCM (0.05)	N/A	TMSOTf (0.5)	-78	N/D
	4	b (2)	DCM (0.05)	N/A	$FeCl_3$ (0.1)	-20	N/D
	5	b (2)	DCM (0.05)	N/A	$FeCl_3$ (0.1)	-60	N/D
b	6	b (1.25)	Tol (0.05)	Ag_2O (1)	TfOH (0.2)	0	20
	7	b (1.25)	Tol (0.05)	Ag_2O (1.5)	TfOH (0.3)	0	35
	8	b (1.25)	Tol (0.1)	Ag_2O (2.5)	TfOH (0.4)	0	60

Conditions Used for FP20 Glycosylation with a Regenerative Glycosylation Protocol

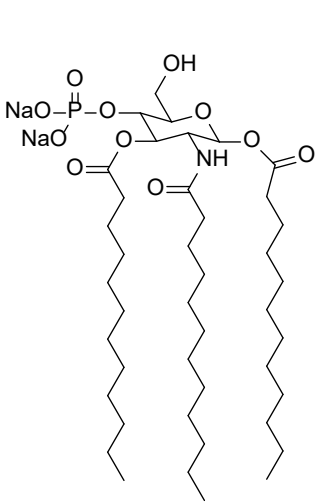
Donors	Entry	Donor (Eq)	Solvent (M)	Promoter (Eq)	Catalyst (Eq)	Time (h)	Yield (%)
a, b	1	a (1.2)	DCM (0.05)	NIS (1.2)	HOFox (0.05)	72	84
	2	a (2.0)	DCM (0.05)	NIS (2.0)	HOFox (0.05)	72	84
	3	b (2.0)	DCM (0.05)	NIS (2.0)	HOFox (0.05)	20	90
c, d	4	c (2.0)	DCM (0.05)	NIS (2.0)	HOFox (0.05)	18	91
	5	d (2.0)	DCM (0.05)	NIS (2.0)	HOFox (0.05)	16	86

Conditions for FP20 Glycosylation Using $Bi(OTf)_3$

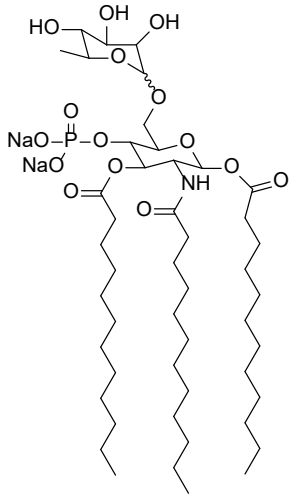
Donors	Entry	Donor (Eq)	Solvent (M)	Promoter (Eq)	Time (h)	Yield (%)
a, b, c, d	1	a (1.2)	DCM (0.05)	$Bi(OTf)_3$ (0.35)	72	45
	2	b (1.2)	DCM (0.05)	$Bi(OTf)_3$ (0.35)	72	46
	3	b (1.5)	DCM (0.05)	$Bi(OTf)_3$ (0.50)	20	60
	4	b (2.0)	DCM (0.05)	$Bi(OTf)_3$ (0.75)	18	84
	5	c (2.0)	DCM (0.05)	Bi(OTf)3 (0.75)	16	84
	6	d (1.25)	DCM (0.05)	$Bi(OTf)_3$ (0.75)	18	94
	7	b (1.25)	DCM (0.05)	$Bi(OTf)_3$ (0.90)	18	80

"A Streamlined Regenerative Glycosylation Reaction: Direct, Acid Free Activation of Thioglycosides", S. Escopy, Y. Singh, K. J. Stine, A. V. Demchenko, Chem. Eur. J.,

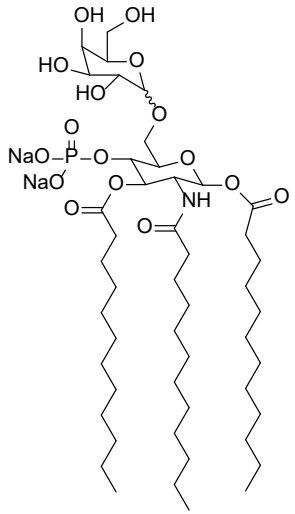
NEW GENERATION OF ANTAGONISTS: BIOLOGICAL ASSESSMENT



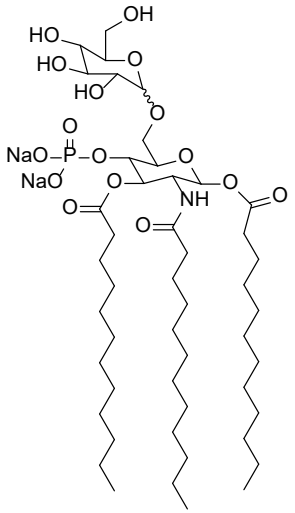
FP20



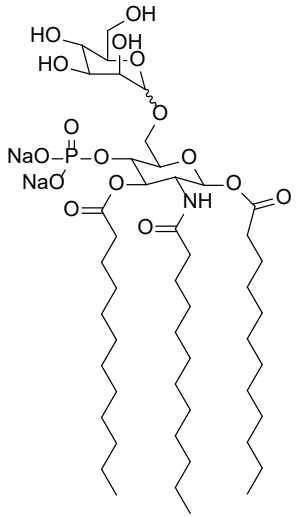
FP20Rha



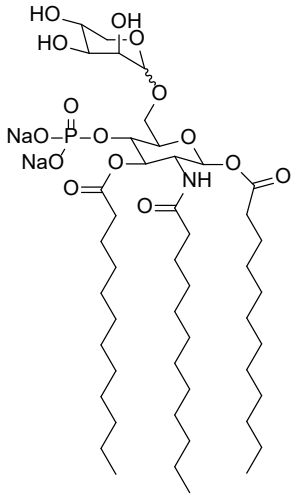
FP20Gal



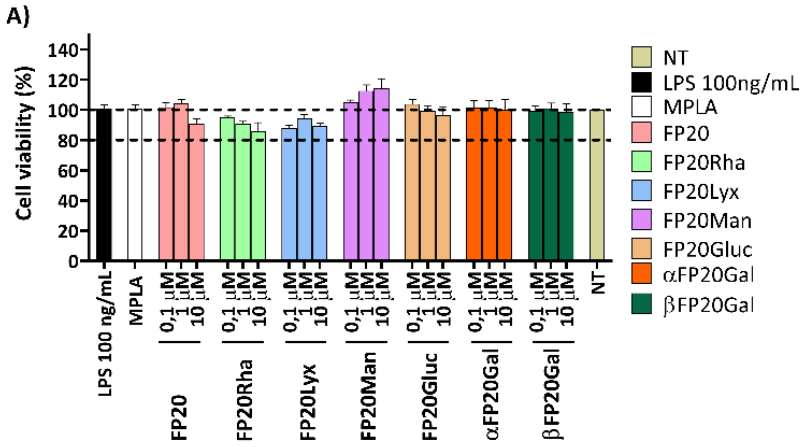
FP20Glc



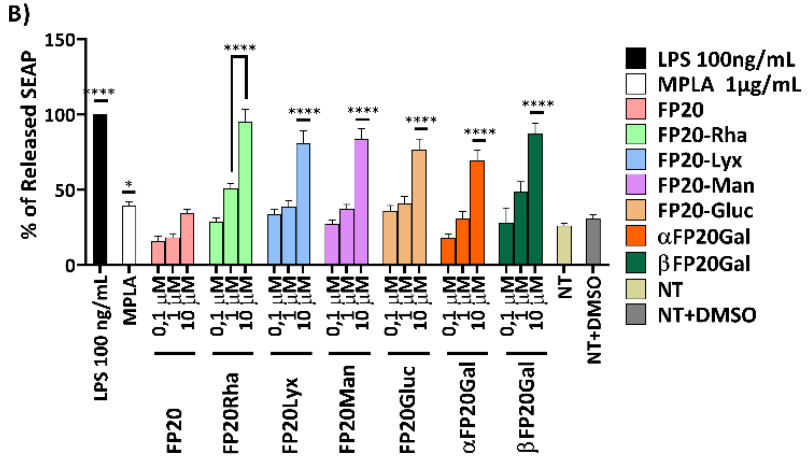
FP20Man



FP20Lyx



Cell viability of THP-1 macrophages analysed by MTT assay.



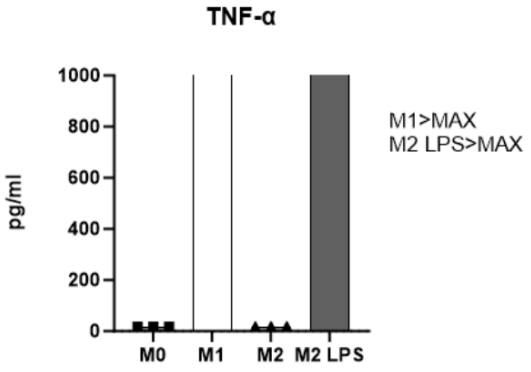
TLR4 antagonism analysed by SEAP translocation in HEK-Blue hTLR4 cells.

FP20 AND GLYOCOSILATED DERIVATIVES WERE PATENTED IN 2021 (**Patent: "IT102021000019544",** A. Romerio, F. Peri, *[2021]*)
LICENSE BOUGHT BY CRODA

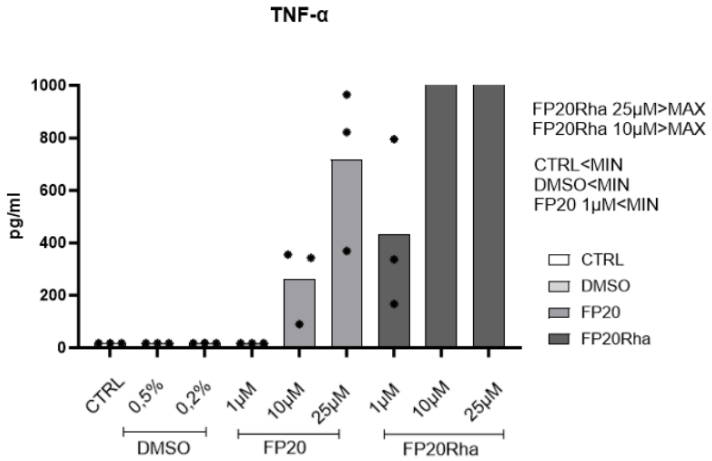
NEW GENERATION OF AGONISTS: ANTICANCER PROPERTIES

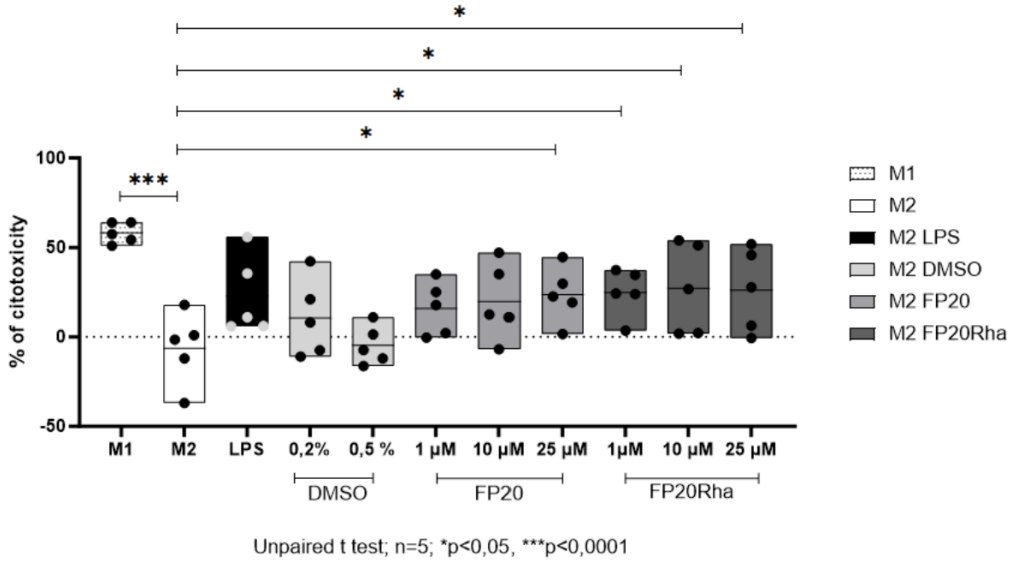
COLLABORATION WITH DR. GIOVANNA D'AMICO'S GROUP IN SAN GERARDO (MONZA)

FP-20 AND FP20Rha POLARIZE MACROPHAGES FROM M2 PHEMOTYPE (PRO CANCER) TOWARDS M1 (ANTI-CANCER) IN LEUKEMIA (697 CELL LINE)



24h





- M1: high TNF-α expression.
- M2: no TNF- α expression.

FP-20 and FP-20Rha (Rhamnosilated derivative) shift TNF-α expression of M2 macrophages towards M1 like expression.

Treatment with FP-20 and FP-20Rha increases macrophages cytotoxicity towards 697 cell line.

ANTIBODY DRUG CONJUGATES

DEVELOPMENT OF AN ANTIBODY-DRUG CONJUGATE (ADC) TO SELECTIVELY DELIVER FP20 AND FP20Rha TO MACROPHAGES IN CANCER ENVIRONMENT

PAYLOAD

- Active principle
- Cytotoxic or immunogenic

ANTIBODY

- Recognize an epitope overexpressed by target cells
- Drives the distribution of the conjugate

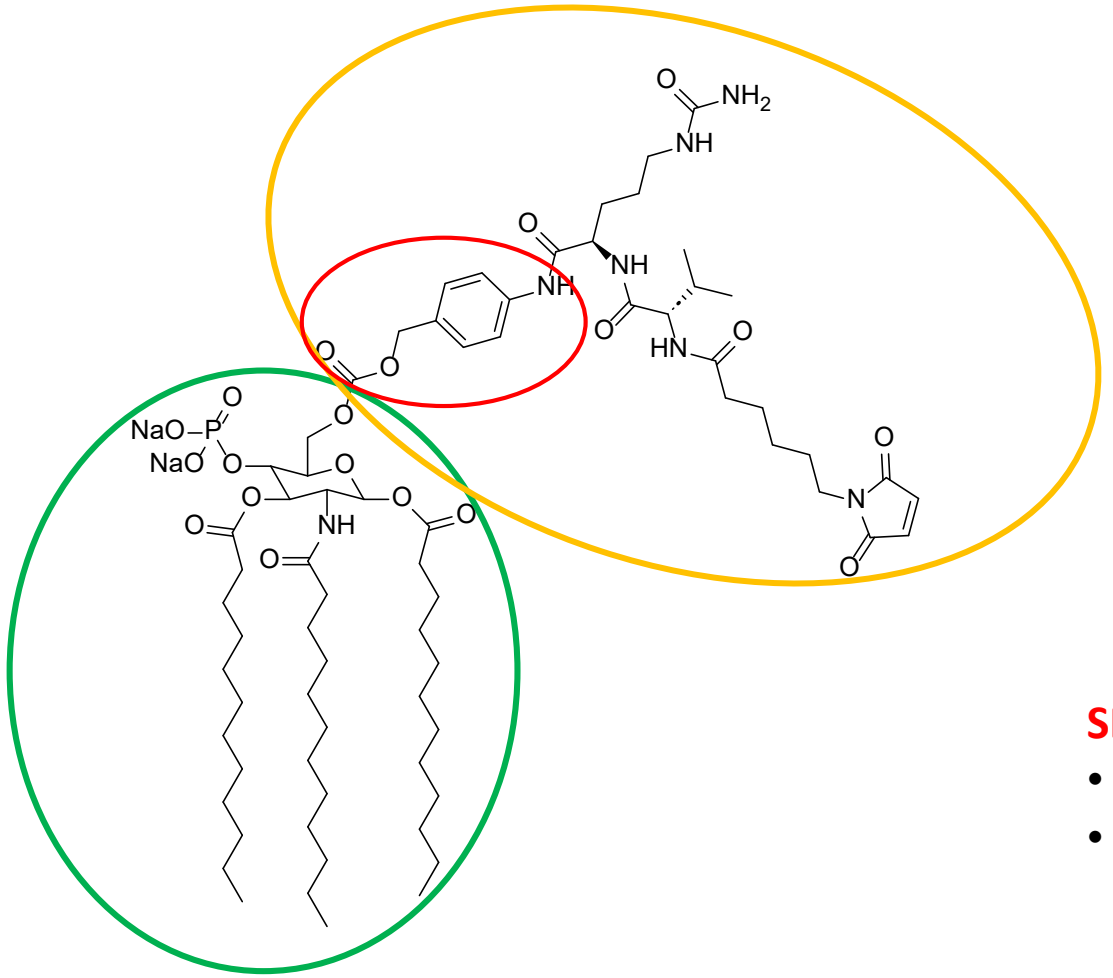
LINKER

- Connects payload to the antibody
- Cleaved only in desired conditions (pH, proteases)
- Could comprise a self-immolative moiety: release of unaltered active principle

DEVELOPMENT OF DRUG-LINKER CONJUGATE

LINKER

- MC-Val-Cit
- Cleavable by Cathepsins



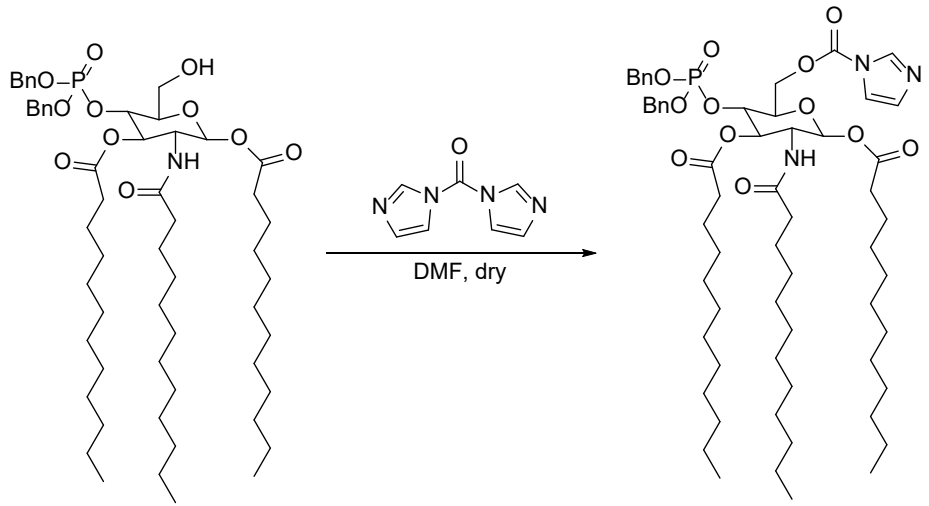
PAYLOAD

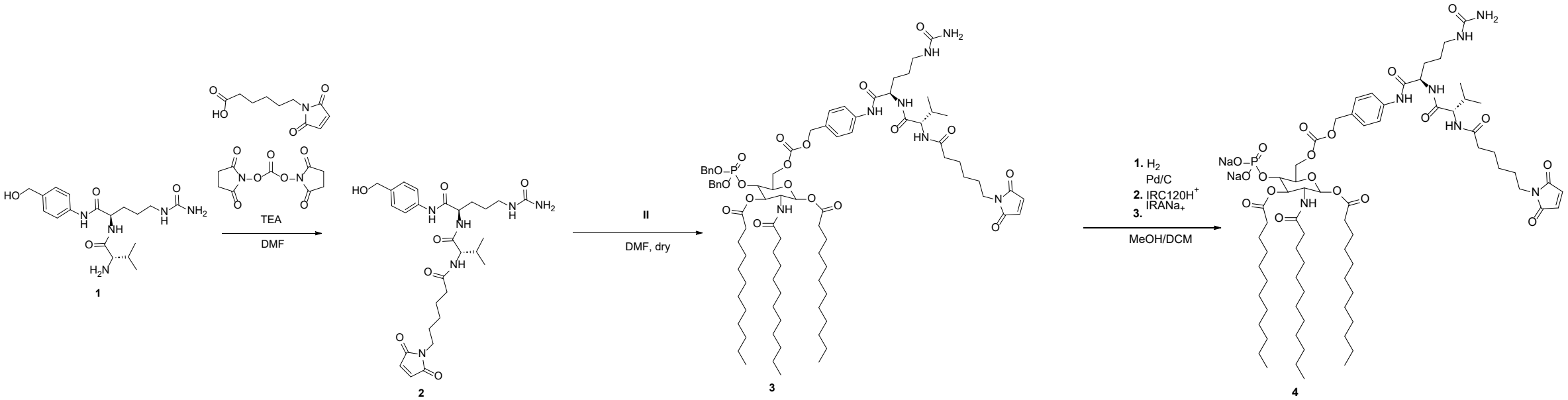
- FP20 or FP20-Rha
- Non cytotoxic payload

SEL IMMOLATIVE MOIETY

- PAB-OH
- Decarboxylate after linker cleavage to release unmodified active principle

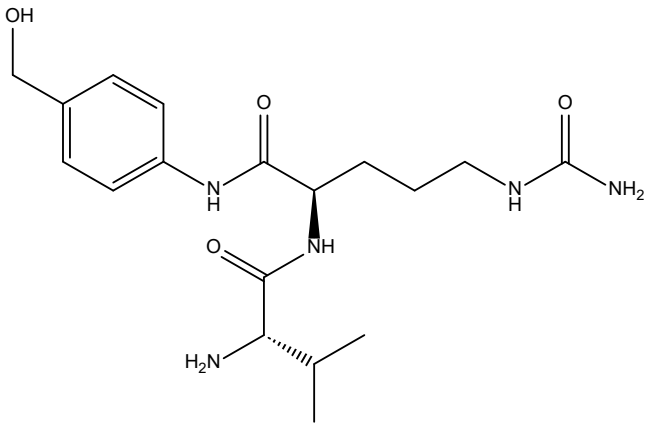
PROPOSED SYNTHESIS OF FIRST DRUG-LINKER CONJUGATE





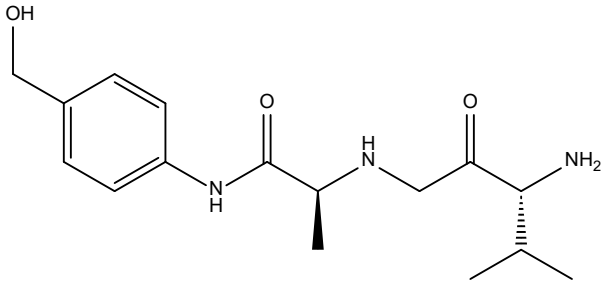
"In vivo assembly of ASGPR binding therapeutics", Patent n°: WO2022035997, Deshpande M.,Saulnier M. G., Sprot K. T., Chen J. J., Ray S., Wiles, J. Allan, *[2022]*

CATHEPSIN CLEAVABLE DIPEPTIDE LINKERS



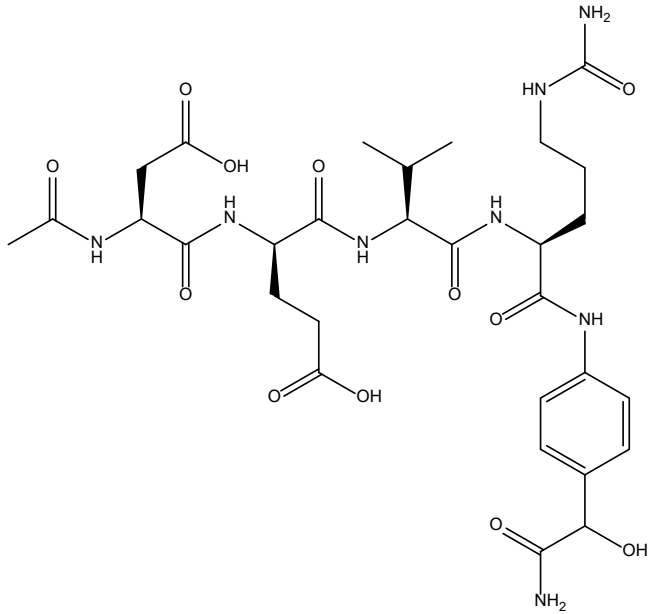
Val-Cit-PAB-OH

- Suitable for internalizing and non-internalizing ADCs. (*Dal Corso 2017*)
- Better release outside membrane when conjugated to IgG Antibody.
- Max DrugAntibody ratio (DAR) (MMAE) = 4



Val-Ala-PAB-OH

- Suitable for internalizing and non-internalizing ADCs. (*Dal Corso 2017*)
- Better activity than Val-Cit.
- Max DAR (MMAE) = 7.4 (Bargh 2019)

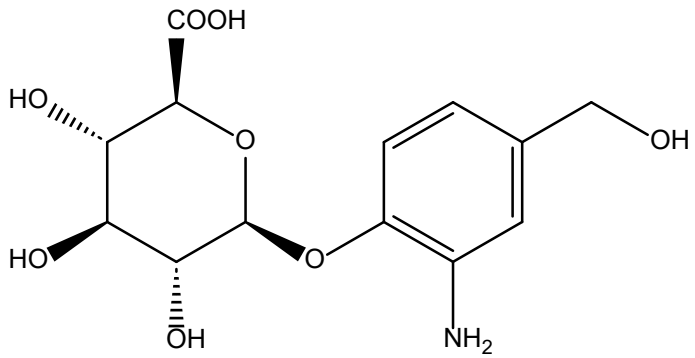


EXO-linker

- Recently developed, never tested for non-internalizing ADCs. (Watanabe 2024)
- More stable in plasma.
- Max DAR (MMAE) = 8

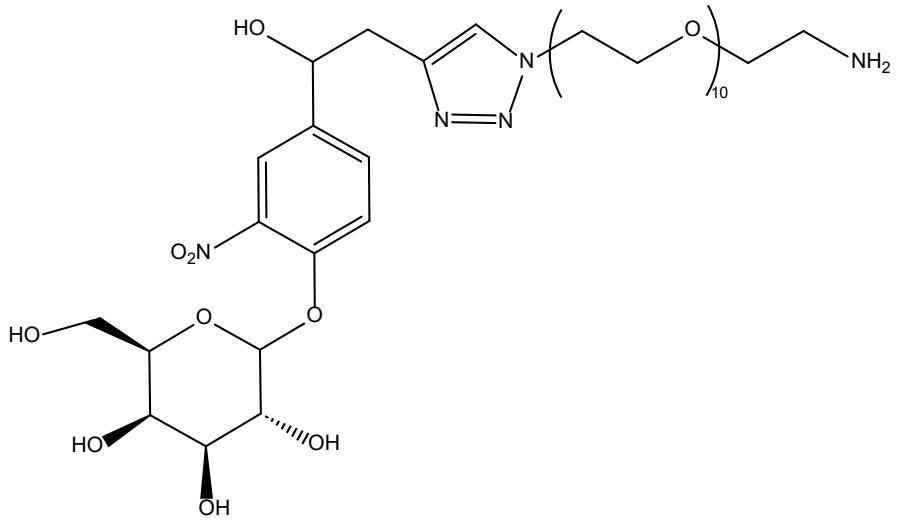
"Protease-Cleavable Linkers Modulate the Anticancer Activity of Non internalizing Antibody−Drug Conjugates" A. Dal Corso, S. Cazzamalli, R. Gebleux, M. Mattarella, D. Neri**,** *Bioconjugate Chemistry,*, **28**, 1826−1833, *[2017]*; **"Exo-Cleavable Linkers: Enhanced Stability and Therapeutic Efficacy in Antibody−Drug Conjugates"** T. Watanabe, N. Arashida, T. Fujii, N. Shikida, K. Ito, K. Shimbo, T. Seki, Y. Iwai, R. Hirama, N. Hatada, A. Nakayama, T. Okuzumi, Y. Matsuda, *J. Med. Chem.*, **67**, 18124−18138 *[2024]*.

GLICOSIDASE CLEAVABLE LINKERS



GLUCORONIC ACID BASED

- Cleaved by Glucoronidase outsode membrane.
- Used in ADCs since 2006.
- DAR (MMAE, MMAF, Doxorubicin) = 8.3

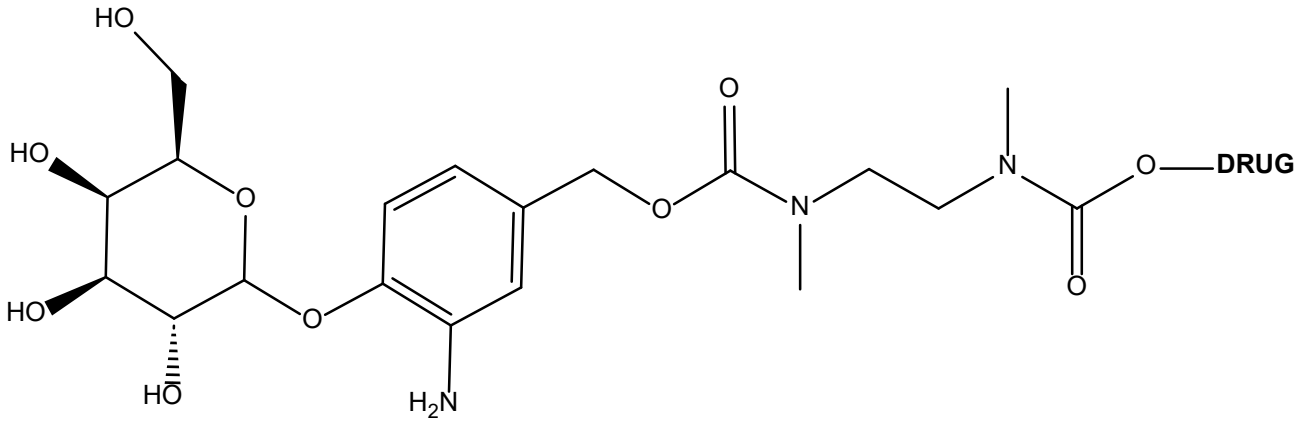


GALACTOSE BASED

- Cleaved by Galactosidase.
- Recently developed. (Kolodych 2017)
- More efficient than the approved drug trastuzumab emtansine (T-DM1) for the treatment of HER2+ mammary tumours in mice.

"Cleavable linkers in antibody–drug conjugates", J. D. Bargh, A. Isidro-Llobet, J. S. Parker , D. R. Spring, *Chem. Soc. Rev.*, **48**, 4361 *[2019],* **"Development and evaluation of b-galactosidase-sensitive antibody drug conjugates",** S. Kolodych, C. Michel,S. Delacroix, O. Koniev, A. Ehkirch, J. Eberova, S. Cianferani, B. Renoux, W. Krezel, P. Poinot, C. D. Muller, S. Papot, A. Wagner, **142,** 376-382, *[2017].*

INNOVATIVE DICARBAMATE FOR ALCOHOL RELEASE

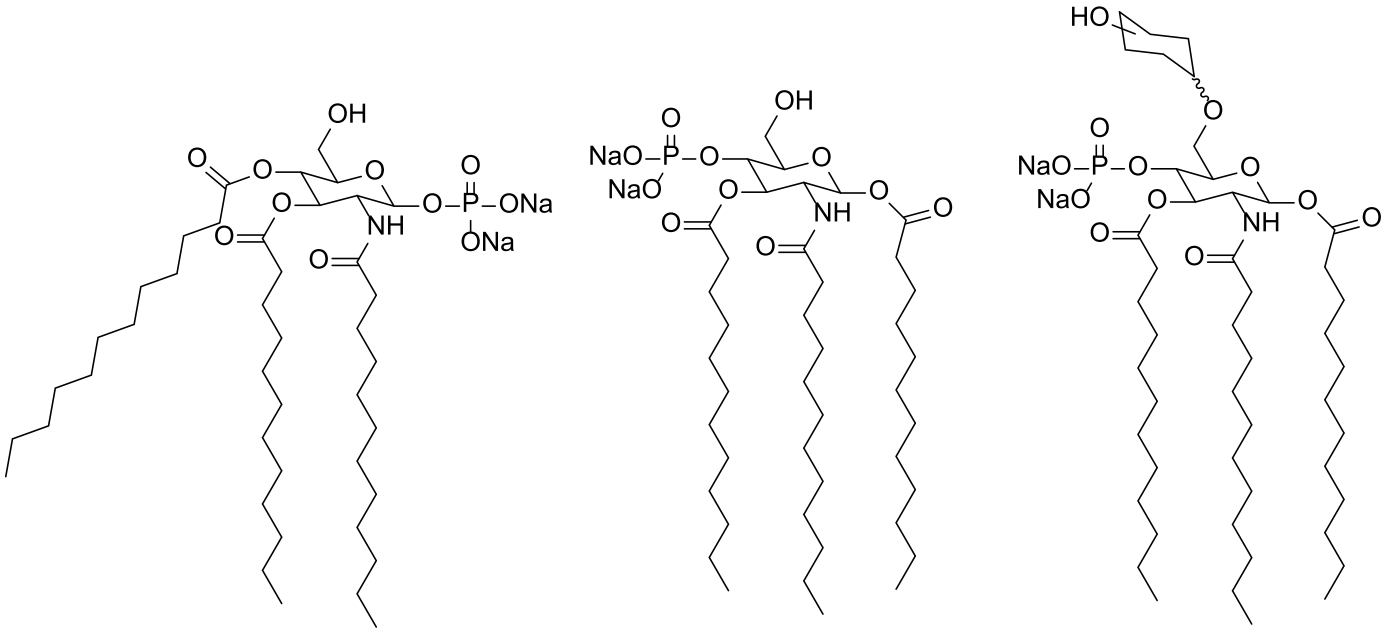


DICARBAMATE SELF-IMMOLATIVE SPACER

- Better and faster release of alcohol conjugated payloads.
- Stable in plasma for 14 days.

"The Methylene Alkoxy CarbamateSelf-ImmolativeUnit:Utilization for the Targeted Delivery of Alcohol-Containing Payloads with Antibody–Drug Conjugates", R. V. Kolakowski, K. T. Haelsig, K. K. Emmerton, C. I. Leiske, J. B. Miyamoto, J. H. Cochran, R. P. Lyon, P. D. Senter, S. C. Jeffrey, *Angew.Chem. Int.*, **55**, 7948–7951, *[2016].*

CONCLUSION AND PERSPECTIVES



FP18 FP20 Glyco-FP20

- **Production of different generations of TLR4 agonists.**
- **New generation is more active than MPLA and LPS but NON toxic.**
- **Able to shift polarization of macrophages' phenotype from M2 to M1.**
- **Possible use as payload in cancer immunotherapy**

FUTURE WORK

- Conjugation of FP20Rha
- Explore functionalization using different linkers.
- Selection of an anti-CD19 antibody to selectively deliver the payload to cancer cells.
- Test on other cancer cell lines.
- Engineering of our own antibody.

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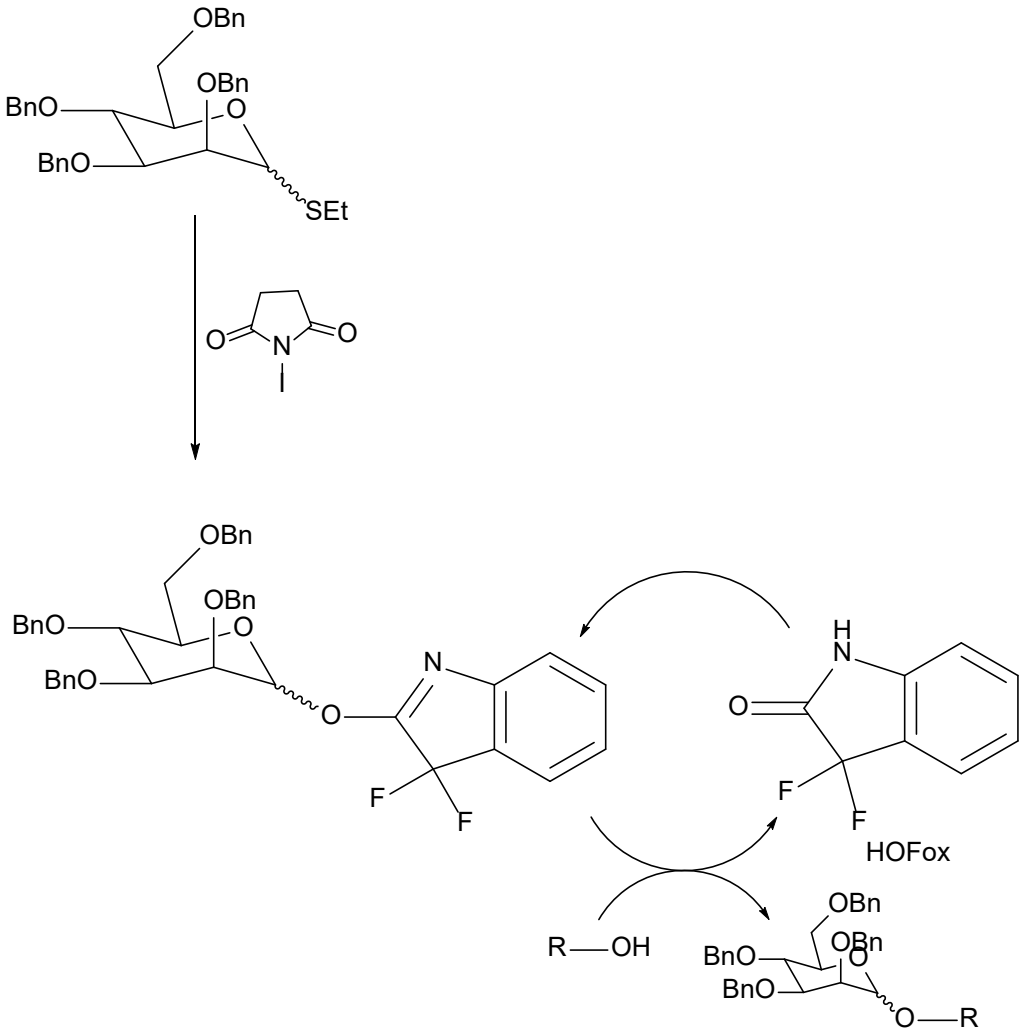
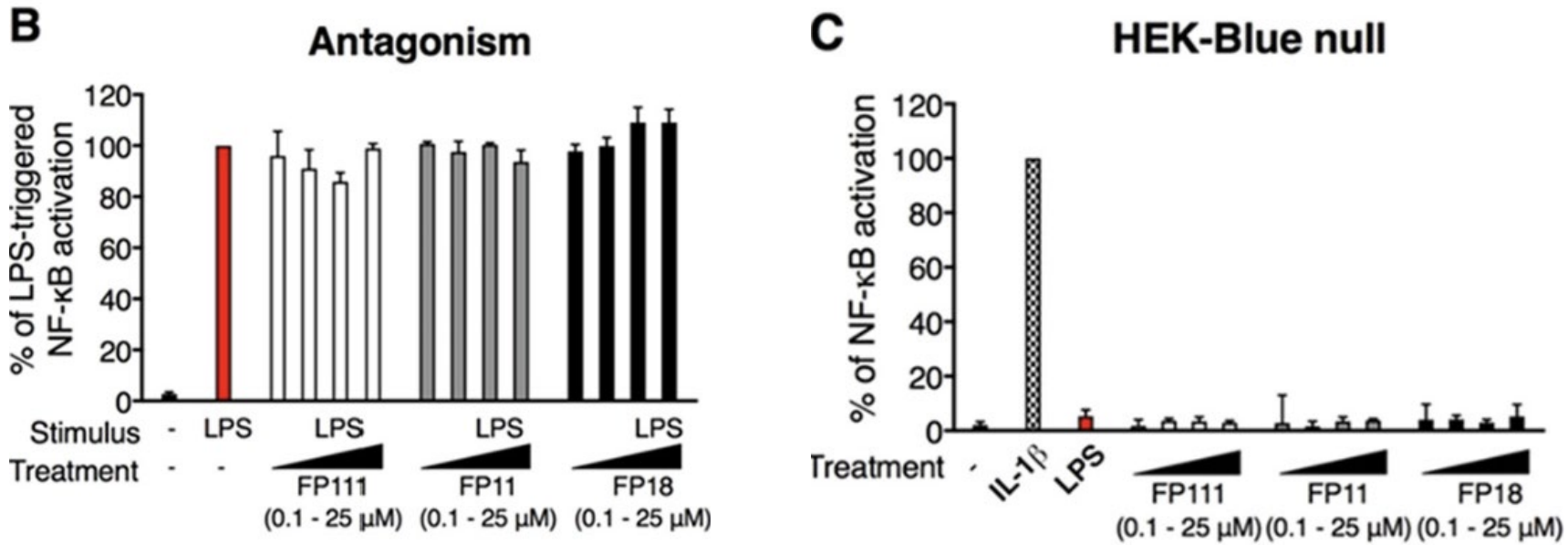
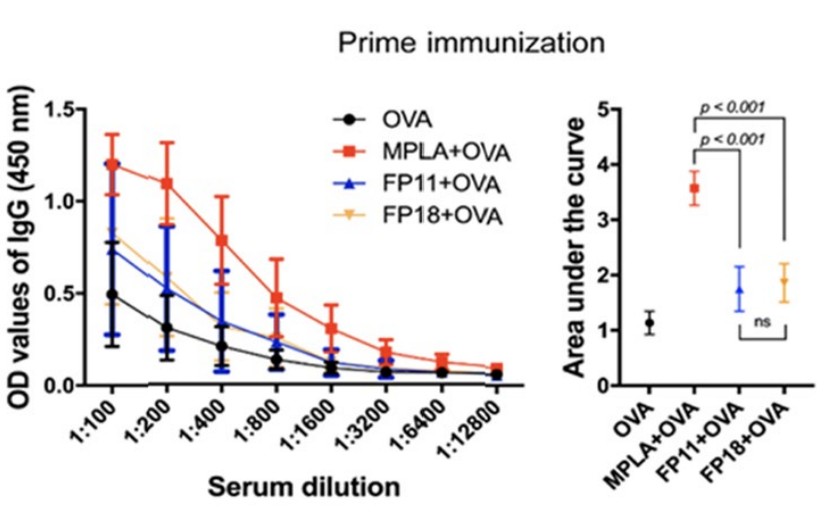
- Sonsoles Martin-Santamaria

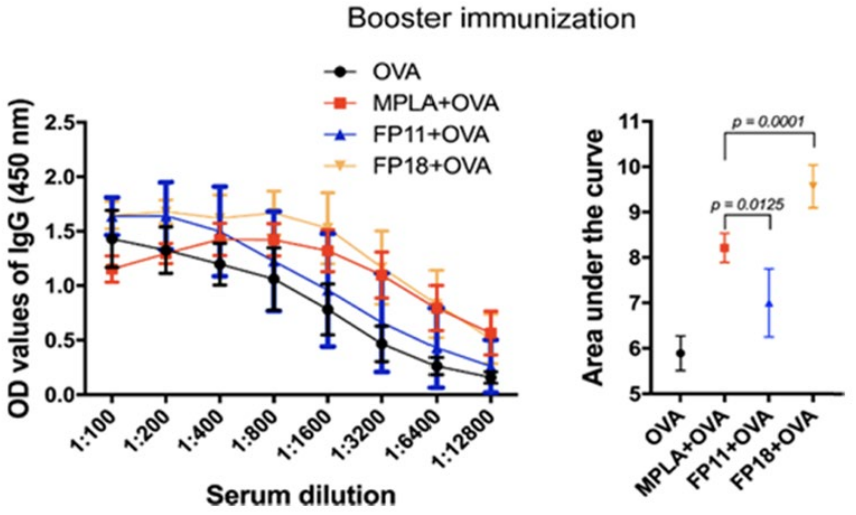
REGENERATIVE GLYCOSILATION MECHANISM




B
Antagonism
% of LPS-triggered NF-κB activation
120
100
80
60
40
20
0
Stimulus - LPS LPS LPS LPS
Treatment - - FP111 FP11 FP18
(0.1 - 25 μM) (0.1 - 25 μM) (0.1 - 25 μM)
C
HEK-Blue null
% of NF-κB activation
120
100
80
60
40
20
0
Treatment - IL-1β LPS FP111 FP11 FP18
(0.1 - 25 μM) (0.1 - 25 μM) (0.1 - 25 μM)

FIRST GENERATION OF AGONISTS: BIOLOGICAL ACTIVITY





IN VIVO STUDIES ON OVOALBUMIN IMMUNIZED MICE

FP18 IS A BETTER ADJUVANT IN VIVO THAN FP11