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SYNTHESIS OF AN ALLOPURINOL RIBOSIDE-MANNOSYLATED POLY-L-LYSINE CONJUGATE

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Abstract : The synthesis of a mannosylated carrier used as a drug delivery system to target specifically an antileishmanial drug allopurinol riboside [4-hydroxy-1-β-D-ribofuranosyl-1H-pyrazolo(3,4-d) pyrimidine] into *Leishmania donovani* infected macrophages *via* their membrane lectin is described. The synthetic construct is made of a poly-L-lysine backbone, partially acylated with δ-gluconolactone to enhance its water-solubility and substituted with glycyl-glycine as a spacer arm. O-*p*-phenylisothiocyanate-5'-phosphodiester derivatives of allopurinol riboside and of inosine were synthesized and attached to the spacer arm, then the remaining spacer arms were substituted by reaction with phenyl acetate mannosyl residues.

Introduction.

The intracellular localization of the pathogen parasite *Leishmania donovani* in macrophages restricts the therapeutic activity of putative antileishmanial drugs. Furthermore, the toxicity of certain drugs precludes use of high doses to kill the parasites.

One way to circumvent both the the lack of bioavailability and the toxicity problem is to use a drug delivery system capable of i) specifically targeting the drugs inside the macrophages in order to reduce the uptake of the drugs by other cells, and ii) releasing the drug in an active form, at proximity of the parasite, to increase the bioavailability of the drug.

Bone marrow-derived macrophages and alveolar macrophages have mannose or fucose receptors[1] which mediate the uptake of macromolecules bearing mannose or fucose residues into acidic endosomes where the ligand is released, carried into lysosomes, and degraded[2]. Using these properties, Monsigny *et al* [3] developed neoglycoproteins which are specifically endocytosed by macrophages, and they showed[4], in *in vitro* experiments, that the capacity of an immunomodulator (muramyl dipeptide, MDP) to activate macrophages is increased at least 100 times when coupled to a mannosylated serum albumin carrier. Subsequently, Roche *et al* [5,6,7] proved in *in vivo* experiments that MDP was active when coupled to such a carrier, whereas free MDP was not.

We have recently developed a new synthetic glycosylated carrier made of a poly-L-lysine (*p*K) backbone, the side chain ε-amino groups of which are partially acylated by δ-gluconolactone while the others are substituted in part by carbohydrate residues and in part by drugs. This synthetic neutral carrier which is easily prepared, highly soluble, readily biodegradable and poorly immunogenic is as efficient as neoglycoproteins to target drugs such as MDP[8]. This delivery system increased 50 times the antiviral activity of phosphonylmethoxyethyladenine (PMEA) in inhibiting the multiplication of herpes simplex virus type 1 in macrophages derived from blood monocytes[9].

To check whether this type of mannosylated polymer is suitable to target an antileishmanial drug (allopurinol riboside, HPP-Rib) to *Leishmania donovani* infected macrophages *via* the mannose-fucose receptor, we undertook the synthesis of O-*p*-phenylisothiocyanate-5'-phosphodiester derivatives of HPP-Rib and of an inactive structural analog used as control, inosine, and their conjugation to the mannosylated poly-L-lysine polymer.

Results and discussion.

The synthesis of the drug carrier conjugate is achieved in steps : 1) synthesis of derivatives of allopurinol riboside and of inosine, 2) substitution of a mannosylated poly-L-lysine carrier with the drugs prepared in step 1.

The carrier synthesis starts from commercially available poly-L-lysine containing an average of 190 lysyl residues per macromolecule. In a first step, a part of the lysyl side chain ε-amino groups are substituted by a Boc-Gly-Gly-OH dipeptide spacer arm to enhance the accessibility of the substituants as shown by Derrien *et al* [8]; the remaining ε-amino groups being acylated by gluconoyl groups which maintain the water-solubility of the polymer and suppress its polycationic character. The Boc protecting groups are further cleaved by an acidic treatment, leaving α-amino groups ready to be substituted with the HPP-Rib, inosine and mannosyl derivatives.

To be coupled to the polymer, HPP-Rib, inosine, and mannosyl derivatives must be modified to present a function able to react with α-amino groups and suitable to allow an efficient release of the active drug inside the cell. We developed the synthesis of O-*p*-isothiocyanatophenyl-5'-phosphodiester derivatives of HPP-Rib and of inosine (Fig. 1 and 2). The isothiocyanate group readily reacts with the polymer α-amino groups, and the phosphodiester linkage can be hydrolyzed by lysosomal acid phosphatase allowing an efficient release of the drug. The number of drugs coupled onto the polymer was determined by U.V. spectrophotometry, and the substitution with mannosyl residues was achieved by coupling on the remaining amino groups a *p*-(carboxymethyl)phenyl-α-D-mannopyranoside derivative of the mannose synthesized as described by Monsigny *et al* [3]. The number of mannose residues linked to the polymer was determined by the resorcinol sulfuric acid method described by Monsigny *et al* [11].

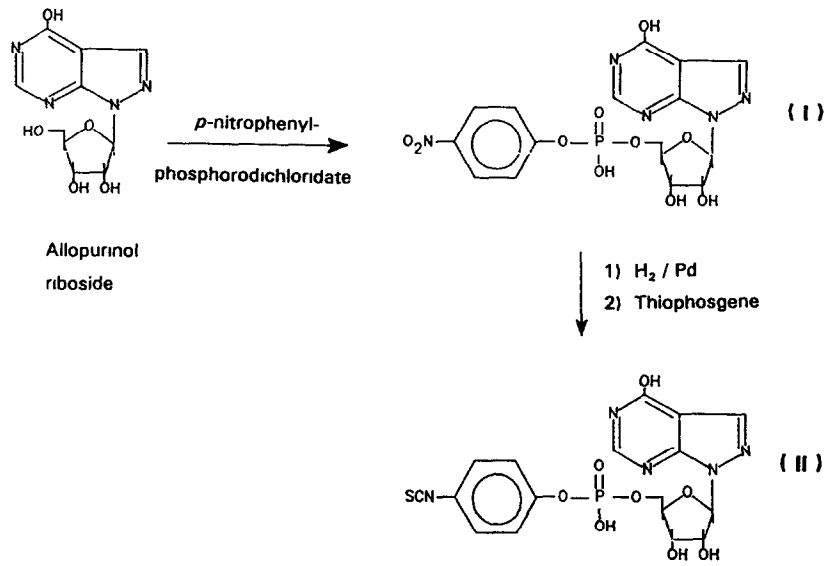

Figure 1: Synthesis of O-*p*-isothiocyanatophenyl-5'-phosphodiester allopurinol riboside.

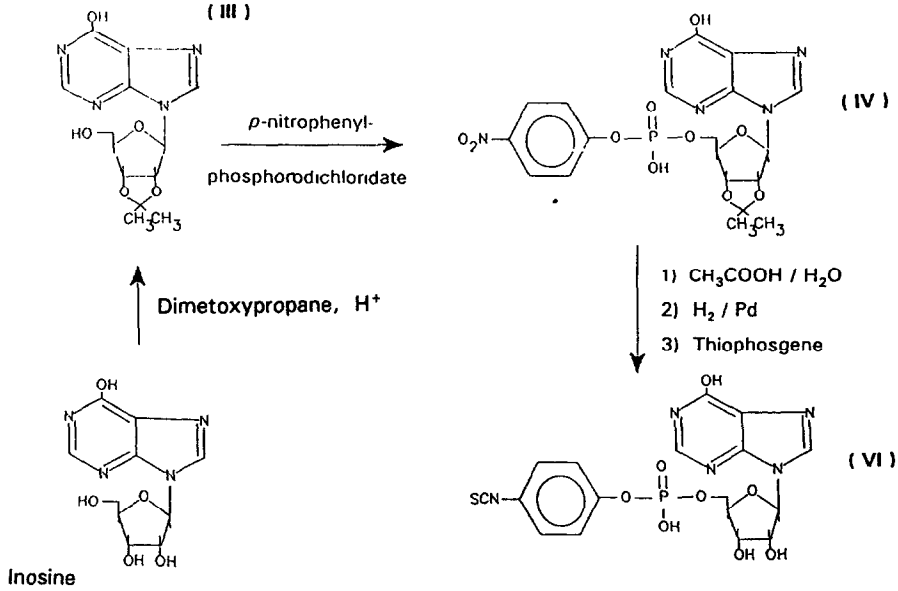

Figure 2 : Synthesis of O-*p*-isothiocyanatophenyl-5'-phosphodiester inosine.

The phenylisothiocyanate phosphodiester derivative of HPP-Rib has been synthesized by the reaction of *p*-nitrophenylphosphorodichloridate on the 5'-hydroxyl group of the drugs according to the method described by Turner and Khorana[12], to give O-*p*-nitrophenyl-5'-phosphodiester allopurinol riboside (HPP-Rib-5'-P-φ-NO_2).

This product was then reduced to O-*p*-aminophenyl-5'-phosphodiester allopurinol riboside (HPP-Rib-5'-P-φ-NH_2) and allowed to react with thiophosgene, as described by Buss and Goldstein[13], to give the O-*p*-isothiocyanatophenyl-5'-phosphodiester allopurinol riboside. The inosine derivative was synthesized in the same way (Fig. 2), starting from 2',3'-O-isopropylidene protected inosine; the isopropylidene protecting group being removed by acidic treatment upon reaction with *p*-nitrophenyl phosphorodichloridate.

The synthesis of the starting gluconoylated carrier Ac_{19}-,$GlcA_{100}$-,$(Tos,H\text{-}GG)_{72}$-*p*K (compound VII) was achieved as previouly described (8, 9). The α-amino group of the glycyl residues is substituted by reaction with O-*p*-isothiocyanatophenyl-5'-phosphodiester allopurinol riboside or inosine derivative. The mannose residues are coupled to remaining amino groups through the carboxyl group of the *p*-(carboxymethyl)phenyl-α-D-mannopyranoside according to the method of Derrien *et al*[8], but using BOP reagent (instead of the classical DCC/ HOBt coupling reagent), which allows the polymer to be solubilized in dimethylsulfoxide, and reduces the coupling reaction time. The general formula of such a *p*K conjugate substituted with *w* acetyl (Ac) , *x* gluconoyl (GlcA) residues, *y* HPP-Rib and *z* mannosyl (Man) residues through a glycyl-glycine spacer arm is : Ac_w-,$(GlcA)_x$-,$(HPP\text{-}Rib\text{-}5'\text{-}P\text{-}\varphi\text{-}TC\text{-}Gly\text{-}Gly)_y$-,$(Man\text{-}Gly\text{-}Gly)_z$-*p*K, where $w+x+y+z=190$, and is schematically shown in Fig. 3.

Ac_w-,$GlcA_x$-,$(Tos,H\text{-}Gly\text{-}Gly)_a$-*p*K **(VI)**

↓ 1) HPP-Rib-5'-P-ϕNCS
↓ 2) Man-ϕ-acetate

Ac_w-,$GlcA_x$-,$(HPP\text{-}Rib\text{-}5'\text{-}P\text{-}\phi\text{-}TC\text{-}Gly\text{-}Gly)_y$-,$(Man\text{-}Gly\text{-}Gly)_z$-*p*K **(X)**

Scheme 1: Strategy of synthesis of allopurinol riboside-, mannosylated-poly-L-lysine conjugates.

The antileishmanial activity of these synthetic conjugates was compared with that of free allopurinol riboside, and with that of Pentostam used as reference positive control, in an *in vitro* model with *Leishmania donovani*-infected murine macrophages[10]. The 50 % effective dose of allopurinol riboside linked to the mannosylated poly-L-lysine was lower than 7.5×10^{-6} M, whereas

it was up to 3 x 10^{-4}M for the free drug, indicating that the drug bound to the polymer was 50 times more active than the free drug.

Using the same strategy, antiviral drugs such as ribavirine have also been modified into O-*p*-isothiocyanatophenyl-5'-phosphodiester derivatives and coupled to these mannosylated poly-L-lysine carriers with the aim to target these drugs into infected macrophages by increasing their bioavailability and reducing their toxicity.

More generally, this kind of synthetic polymer could be suitable to target other drugs to different kind of cells according to the recognition ligands bound to the polymer.

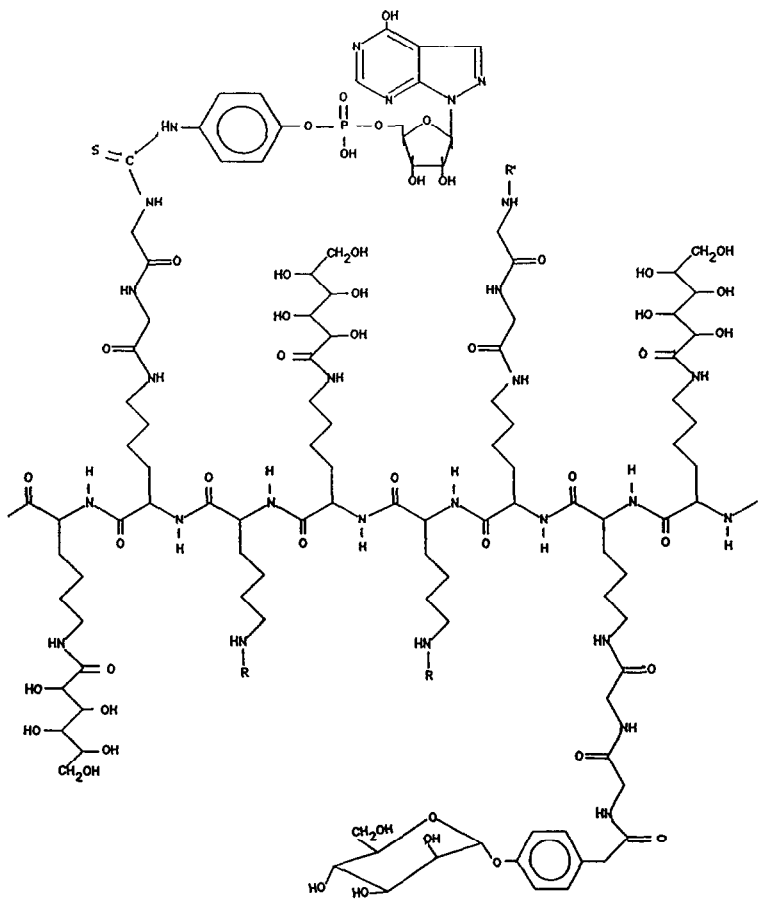

Figure 3: Structure of the conjugate Ac_w-,$(GlcA)_x$-,(HPP-Rib-5'-P-φ-TC-Gly-Gly)$_y$-,$(Man\text{-}Gly\text{-}Gly)_z$-*p*K. R = GlcA or R'-Gly-Gly and R' = Ac, HPP-Rib-5'-P-φ-TC or Man, and w + x + y + z = 190)

Experimental section

Chemical methods

Poly-L-lysine,HBr (ρK) (30,000-50,000) containing about 190 lysine residues and N_α-Boc-glycyl-glycine (N_α-Boc-Gly-Gly-OH) were purchased from Bachem Feinchemikalien (Bubendorf, Switzerland); D-gluconic acid δ-lactone was obtained from Roquettes Frères (Lestrem, France); acetic anhydride, p-toluenesulfonic acid monohydrate from Merck (Darmstadt, Germany); *N*-ethyldiisopropylamine (DIEA), 4-dimethylaminopyridine (DMAP), p-nitrophenyl phosphorodichloridate (p-$NO_2\phi$-P-Cl_2) and thiophosgene from Aldrich (Steinheim, Germany); benzo-triazol-1-yl-oxy-tris (dimethylamino) phosphonium, hexafluoro-phosphate (BOP reagent) from Richelieu Biotechnologies (St-Hyacinthe, Canada). Allopurinol riboside (HPP-Rib) and inosine (I), were obtained from Sigma (St Louis, MO, U.S.A). p-(carboxymethyl) phenyl-tetra-acetyl-α-D-mannopyranoside was synthesized as previously described by Derrien *et al*[8], according to the procedure of Helferich and Schnitz-Hillebrecht[14].

Thin layer chromatographies (TLC) analyses were performed on pre-coated plastic sheets silica gel 60 F_{254} (Merck, Darmstadt, Germany) using the following solvents: A) $CHCl_3$/MeOH/H_2O (6/6/1, v:v:v); B) $CHCl_3$/MeOH (1/1, v:v); C) $CHCl_3$/MeOH (6/2, v:v); D) $CHCl_3$/MeOH/H_2O/CH_3COOH (6/6/1/0.2, v:v:v:v). Column chromatographies were made with Kieselgel 60, 70-230 mesh (Merck, Darmstadt, Germany). Substituted polymers were purified by gel filtration on Trisacryl GF 0.5 (Sepracor, Villeneuve La Garenne, France). ^{1}H-NMR spectra, recorded at 300 MHz using a Bruker AM 3000 spectrometer interfaced with an Aspect-3000 computer, are expressed as δ units (parts per million) relative to tetramethylsilane used as an internal reference. Amino acid analyses were made using the standard conditions with a Biotronic LC 6000E amino acid analyzer. Infrared spectra were recorded on a Perkin-Elmer 457 spectrophotometer.

1- Synthesis of allopurinol riboside and inosine derivatives.

O-p-isothiocyanatophenyl-5'-phosphodiester allopurinol riboside (HPP-Rib-5'-P-ϕNCS) was synthesized by reaction of HPP-Rib with p-nitrophenylphosphorodichloridate to give O-p-nitrophenyl-5'-phosphodiester allopurinol riboside (HPP-Rib-5'-P-ϕNO_2). This product was then reduced to O-p-aminophenyl-5'-phosphodiesterallopurinolriboside (HPP-Rib-5'-P-ϕNH_2), which was further reacted with thiophosgene to give the p-isothiocyanatophenyl-5'-phosphodiesterderivative of allopurinol riboside . The inosine derivative was prepared by the same way, starting from O-2',3'-isopropylidene inosine synthesized according to Fuertes *et al*[15].

O-*p*-Nitrophenyl-5'-phosphodiester allopurinol riboside (HPP-Rib-5'-P-ϕNO_2) (I).

To a stirred solution of allopurinol riboside (50 mg, 0.186 mmol) in anhydrous pyridine (2 ml) a solution of *p*-nitrophenylphosphorodichloridate (52.6 mg, 0.186 mmol) in dry pyridine (2 ml) was rapidly added and the mixture was stirred for 1 h at 20°C. TLC analysis in solvent (A) showed a complete disparition of allopurinol riboside R_f(B) 0.62, R_f(A) 0.85. A solution of 10% water in pyridine (10 μl) was added under stirring. The mixture was then poured into cold diethylether, filtered and dried *in vacuo*. The compound was purified by chromatography on a silicagel column in the solvent (A). Yield 46.1 mg (56 %) R_f(A) 0.68 ; R_f(B) 0.31. The presence of *p*-nitrophenyl group was evidenced by UV at 274 nm. ^{1}H-NMR (DMSO-d6) δ=3.85 (1H, m, H'$_4$), 4.05 (2H, m, H'$_5$), 4.28 (1H, m, H'$_3$), 4.55 (1H, m, H'$_2$), 6.09 (1H, d, H'$_1$), 7.30 (2H, d, $H_{2,6}\phi NO_2$), 8.04 (2H, d, $H_{3,5}\phi NO_2$), 8.08 (1H, s, H_2), 8.11 (1H, s, H_7).

O-*p*-isothiocyanatophenyl-5'-phosphodiester allopurinol riboside (HPP-Rib-5'-P-ϕNCS), II.

I (46.1 mg, 0.1 mmol) was dissolved in 4 ml of a water/ethanol mixture (1/1, v:v) and hydrogenolyzed over activated charcoal (10% Pd) during 1 h at 20°C. The conversion of I into O-*p*-aminophenyl-5'-phosphodiester allopurinol riboside was complete as shown by TLC analysis in solvent A.. The solution was filtered and thiophosgene (1.5 μl, 0.2 mmol) was added. The reaction mixture was stirred for one hour at
20°C, leading to a complete conversion of *p*-aminophenyl allopurinol riboside R_f(B) 0.23 into II R_f(B) 0.40. The solution was evaporated to dryness under reduced pressure and the residual solid was dissolved in water and freeze-dried, yield 28.1 mg (63%). The isothiocyanate group was evidenced by IR spectroscopy at 2100 cm^{-1}.

2',3'-O-isopropylidene inosine, III.

To a solution of inosine (2.0 g, 7.5 mmol) in dry acetone (50 ml), *p*-toluenesulfonic acid (1.42 g, 7.5 mmol) and dimethoxypropane (3 ml, 37.3 mmol) were added. After 2 h stirring at 20°C, the conversion of inosine R_f(C) 0.16 into inosine-2,'3'-isopropylidene R_f(C) 0.70, which precipitates, was complete. The product was collected by centrifugation, washed twice with acetone, and finally with diethylether. The product was filtered off, dried *in vacuo* and purified by chromatography on a silica gel column eluted with solvent C, yield 1.40 g (60%). R_f(B) 0.88. ^{1}H-NMR (DMSO-d6) δ= 1.34 and 1.50 (3H each, 2s, CH3), 3.52 (2H, d, H'$_5$), 4.21 (1H, m, H'$_4$), 4.93 (1H, m, H'$_3$), 5.26 (1H, m, H'$_2$), 6.09 (1H, d, H'$_1$), 8.07 and 8.28 (1H each, 2s, H_2 and H_8).

O-*p*-Nitrophenyl-5'-phosphodiester-2',3'-O-isopropylidene inosine, IV.

To *p*-nitrophenyl phosphoro-dichloridate (85 mg, 0.32 mmol) dissolved in anhydrous pyridine (1 ml), a solution of III (100 mg, 0.32 mmol) in pyridine (1ml) was added dropwise and the solution

was stirred for 1 h at 20°C. Inosine isopropylidene [R_f(B) 0.88] was quantitatively converted into **II** R_f(B) 0.40. A solution of 10% water in pyridine (57.6 μl) was added rapidly with stirring and the mixture was poured in cold diethylether, filtered and dried *in vacuo*. The compound **IV** was further purified by chromatography on a silica gel column eluted with the solvent B, yield 75 mg (47%). ^{1}H-NMR (DMSO-d6) δ = 1.23 and 1.50 (3H each, 2s, CH3), 3.86 (2H, m, H'$_5$), 4.30 (1H, m, H'$_4$), 4.85 (1H, m, H'$_3$), 5.21 (1H, m, H'$_2$), 6.05 (1H, d, H'$_1$), 7.35 (2H, d, $H_{2,6}$ ϕNO_2), 8.12 (2H, d, $H_{3,5}$ ϕNO_2), 8.03 and 8.29 (1H each, 2s, H_2 and H_8).

O-*p*-Nitrophenyl-5'-phosphodiester inosine (IMP-ϕNO_2), V.

After 10 h stirring at 60°C of a solution of **IV** (75 mg, 0.15 mmol) in 4 ml acetic acid/water mixture (1/1, v:v), **IV** [R_f(B) 0.40] was quantitatively converted into O-*p*-nitrophenyl-5'-phosphodiester inosine [R_f(B) 0.23]. Acetic acid was then removed under reduced pressure at 20°C and the product was freeze-dried to give 70 mg (100%). The compound was further characterized by ^{1}H-NMR (DMSO-d6). δ = 3.85 (2H, m, H'$_5$), 3.98 (1H, m, H'$_4$), 4.21 (1H, m, H'$_3$), 4.40 (1H, m, H'$_2$), 6.10 (1H, d, H'$_1$), 7.38 (2H, d, $H_{2,6}$ ϕNO_2), 8.15 (2H, d, $H_{3,5}$ ϕNO_2), 8.09 and 8.30 (1H each, 2s, H_2 and H_8).

O-*p*-isothiocyanatophenyl-5'-phosphodiester inosine (IMP-ϕNCS), VI.

O-*p*-Isothiocyanatophenyl-5'-phosphodiesterinosine was prepared in the same way as described above for the synthesis of the allopurinol riboside derivative. Yield 48.5 mg (66%).

2- Synthesis of the conjugate Ac_{19}-,$GlcA_{100}$-,(HPP-Rib-5'-P-ϕTC-Gly-Gly)$_{16}$-,(Man-Gly-Gly)$_{52}$-*p*K.

The poly-L-lysine carrier Ac_{19}-,$GlcA_{100}$-,(Tos,H-GG)$_{72}$-*p*K **(VII)** was synthetized as previouly described (9).

Ac_{19}-,$GlcA_{100}$-,(HPP-Rib-5'-P-ϕTC-Gly-Gly)$_{16}$-*p*K, VIII.

To a solution of **VII** (160 mg, 2.53 μmol) in 5 ml of a DMF/DMSO mixture (1/1, v:v), DIEA (39.1 μl, 0.22 mmol) and **II** (28.1 mg, 0.63 mmol) were successively added and the mixture was stirred at room temperature. The coupling reaction was complete within 2 h. The polymer was collected as described above to yield 160 mg (92 %). The number of bound HPP-Rib, determined by absorbance at 251 nm, was 16 per macromolecule.

Ac_{19}-,$GlcA_{100}$-,(HPP-Rib-5'-P-ϕTC-Gly-Gly)$_{16}$-,(Man-Gly-Gly)$_{52}$-*p*K, IX.

To a solution of **VIII** (80 mg, 1.17 μmole) in 5 ml of a DMF/DMSO mixture (1/1, v:v) Man ϕ-acetate (25.4 mg, 0.075 mmol), DIEA (40.0 μl, 0.23 mmol) and BOP (50 mg, 0.11 mmol) were

added. The reaction was essentially quantitative within 2 h at 25°C, as shown by the total disappearance of the mannoside [R_f(D) 0.77]. Acetic anhydride (2 µl, 0.02 mmol) was then added and the acylation of the residual α-amino groups of glycine was complete within 20 min. The polymer was collected as described above and further purified by gel filtration on a Trisacryl GF 0.5 column (20 x 2.5 cm) equilibrated with H_2O/n-butanol (95/5, v:v), yield 85 mg (97%). The sugar content, determined by the resorcinol sulfuric acid method[11], showed that a macromolecule bears 52 mannose residues.

Synthesis of Ac_{19}-,$GlcA_{100}$-,(HPP-Rib-5'-P-φTC-Gly-Gly)$_{16}$-,(Ac-Gly-Gly)$_{56}$-pK, X.

To a solution of **VIII** (80 mg, 1.17 µmole) in 5 ml of a DMF/DMSO mixture, DIEA (30.5 µl, 0.175 mmol) and acetic anhydride (7.0 µl, 0.07 mmol) were successively added. The solution was stirred 20 min at 20°C when a complete acetylation of residual amino groups was achieved. The sugar free control polymer was then collected and purified as described above.

The synthesis of Ac_{19}-,$(GlcA)_{100}$-,(IMP-φTC)$_{17}$-,(Man-Gly-Gly)$_{51}$-,(Ac-Gly-Gly)$_4$-pK, and of Ac_{19}-,$(GlcA)_{100}$-, (IMP-φTC)$_{17}$-,(Ac-Gly-Gly)$_{56}$-pK were achieved and characterized as described above, the number of bound IMP, determined by absorbance at 256 nm, was found to be 17 per macromolecule.

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References

1. Stahl, P.; Gordon, S. *J.Cell Biol.* **1982**, *93*, 49-56.
2. Wileman,T.; Harding,C.; Stahl, P. *Biochem. J.* **1985**, *232*, 1-14.
3. Monsigny, M.; Roche, A.C.; Midoux, P. *Biol. Cell.* **1984**, *51*, 187-196.
4. Monsigny, M.; Roche, A.C.; Bailly, P. *Biochem. Biophys. Res. Commun.* **1984**, *121*, 579-584.
5. Roche, A.C.; Bailly, P.; Monsigny, M. *Invasion Metastasis.* **1985**, *5*, 218-232.
6. Roche, A.C.; Midoux, P.; Petit, C.; Derrien, D.; Mayer, R.; Monsigny, M. *Adv. Biosci.* **1988**, *68*, 217-235.
7. Roche, A.C.; Monsigny, M. *Ann. Inst. Pasteur Immunol.* **1986**, *137C*, 223-226.
8. Derrien, D.; Midoux, P.; Petit, C.; Nègre, E.; Mayer, R.; Monsigny, M.; Roche, A.C. *Glycoconjugate J.*, **1989**, *6*, 241-255.
9. Midoux, P.; Nègre, E.; Roche, A.C.; Mayer, R.; Monsigny, M.; Balzarini, J.; De Clercq, E.; Mayer, E.; Ghaffar, A., Gangemi, J.D. *Biochem. Biophys. Res. Commun.* **1990**, *167*, 1044-1049.
10. Nègre, E.; Chance, M.L.; Hanboula, S.Y.; Monsigny, M.; Roche, A.C.; Mayer, R.; Hommel, M. *Antimicrob. Agents Chemother.* **1992**, *36*, 2228-2232.
11. Monsigny, M.; Petit, C.; Roche, A.C. *Anal. Biochem.* **1988**, *175*, 525-530.
12. Turner, A.F.; Khorana, H.G. *J. Am. Chem. Soc.* **1959**, *81*, 4651-4656.
13. Buss, D.H.; Goldstein, I.J. *J. Chem. Soc.* **1968**, 1457-1461.
14. Helferich, B.; Schmitz-Hillebrecht, E. *Ber. Dtsch. Chem. Ges.*, **1933**, *121*, 231-238.
15. Fuertes, M.; Witkowski, J.T.; Steeter, D.G., Robins, R.K. *J. Med. Chem.* **1974**, *17*, 642-645.
16. Fields, R. *Biochem. J.* **1971**, *124*, 581-590.