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Synthesis of Mannose-6-Phosphate Analogues and their Utility as Angiogenesis Regulators


Although carbohydrates are the most abundant natural products, their use as therapeutic agents has been limited. However, since carbohydrate binding proteins are involved in many biological processes, including cellular communication,[1–2] the prospects for carbohydrate-based drugs seem bright. Here, we provide a synthetic route to bioactive mannose derivatives that serve as both positive and negative effectors of angiogenesis, thereby laying the groundwork for future drug development.

The current, limited applications of carbohydrates as therapeutics may, in part, be related to the high complexity of interactions between carbohydrate and carbohydrate binding proteins. Carbohydrate oligomers are themselves complex; for example, four different monosaccharides can form 35 560 distinct tetrasaccharides—this large number reflects the multiple hydroxy attachment sites on each component sugar. Thus, a relatively small polysaccharide has an enormous capacity to encode biological information. When these polysaccharides are conjugated to proteins, the complexity further increases. To date, more than 80 carbohydrate binding proteins have been identified, and their binding specificities have been described (or are about to be).[3] Among these proteins, the lectin family has been extensively studied and classified into subfamilies according to their cellular location and their carbohydrate binding specificities.[4] For example, the P-type lectins recognize mannose-6-phosphate (M6P), the motivation behind efforts in the design and synthesis of new M6P analogues.

P-type lectins encompass the 46 kDa cation-dependent M6P receptor (CD-M6PR), the 300 kDa cation-independent M6P receptor (CI-M6PR), and proteins harboring M6P homology domains.[5] One major cellular function of the receptors is to help cargo M6P-containing proteins between various subcellular compartments.[6] In addition, CI-M6PR is actually a large multi-compartments.

Current routes to M6P analogues exploit activating the C-6 partner receptor.[7–9] It binds insulin-like growth factor-2 (IGF-2) at the cell surface and internalizes this growth factor for degradation inside lysosomes. The functions of other proteins harboring M6P residues are also dependent on their interactions with this receptor. For example, latent transforming growth factor-β (TGF-β) is converted into active TGF-β upon M6P interaction with the receptor.[7] Leukemia inhibitory factor (LIF), a growth factor, is cleared from the extracellular medium via receptor interaction with M6P, and subsequent internalization prevents an excessive accumulation that is detrimental to health especially during development.[10] Moreover, proliferin-dependent angiogenesis also requires CI-M6PR.[11] The obvious importance of M6P in biological processes, including angiogenesis, prompted us to evaluate the activity of our new analogues in blood vessel formation. As will be shown, the analogues do indeed have substantial effect on angiogenesis.

The monosaccharides synthesized and examined here include a variety of substituents at the mannose 6-position: azido (4), aminomethyl (6), carboxyl (7), malonate (8), sulfonate (9), carboxymethyl (10), and phosphonate (3). The replacement of the phosphate head group by other moieties, mostly bioisosteres of phosphate, are intended to provide enzymatically stable compounds that could be used as tools to better understand the chemical factors involved in the modulation of angiogenic activities. In the past, the development of carbohydrates for therapeutic purposes has been considered problematic due to the challenges in synthesizing carbohydrate mimics; however, recent progress in this area has allowed us to overcome these limitations. We now present a simple and efficient synthesis of seven M6P analogues using a cyclic sulfate intermediate.

Current routes to M6P analogues exploit activating the C-6 position by a variety of methods, such as halogenation, sulfonation, epoxidation and the use of phosphonate salts followed by nucleophilic substitution.[12–19] Homologation of mannose has been achieved previously by oxidation of the primary alcohol followed by a Wittig–Horner reaction on the resultant aldehyde.[20] Recently, we introduced substitutions at the C-6 position of mannose via a Mitsunobu reaction.[21] These approaches can be efficient, but a need for a more versatile method led us to access such compounds via the cyclic sulfate method previously developed by us[22] to obtain, in just a few steps with the potential for scale-up, a wide variety of C-6 mannose derivatives (Scheme 1).

Regioselective nucleophilic displacement of cyclic sulfate 2 is the key step to obtain all the described mannose derivatives. In the case of phosphonate 3, malonate 8, and sulfonate 9, the corresponding nucleophiles were prepared with the aid of a strong base such as n-butyllithium or sodium hydride. An
Angiogenesis is a complex phenomenon that leads to the formation of new blood vessels from pre-existing ones. This process is crucial for development and plays a key role in various normal and pathological states, including cancer and cardiovascular diseases. Its regulation involves a tuned balance of proangiogenic and antiangiogenic factors. The relationship between M6P and angiogenesis appears firmly established, through both the transforming growth factor (TGF-β) pathway activation and the proliferin (PLF) signal. TGF-β pathways or ligands are thought to have both pro- and antiangiogenic properties. Low TGF-β levels contribute to an angiogenic switch by up-regulating angiogenic factors and proteinases. On the other hand, high TGF-β levels inhibit endothelial cell growth, stimulate smooth muscle cell differentiation, and recruit and promote basement membrane reformation. Regarding the proliferin signal, M6P completely blocks PLF-induced angiogenesis both in cell culture and in vivo. Finally, the receptor itself can affect angiogenesis by clearing active plasminogen through its soluble form, and this has been shown to block tumor cell invasion in vitro, endothelial cell invasion in vivo, and tumor growth in vivo.

The M6P analogues presented in Scheme 1 were subjected to angiogenic assays using two experimental models. The first of these employed the rat aortic ring assay, an ex vivo angiogenic model in which our analogues were examined at 10^{-6} M over 11 days for their ability to stimulate or inhibit capillary growth in rat aortic rings. Sunitinib (marketed by Pfizer as Sutent and formerly known as SU11248) and endothelial cell growth supplement (ECGS) were used as known negative and positive stimuli, respectively. The results are shown in Table 1. Certain derivatives behave as inhibitors: MeM6P, malonate, and phosphonate, while others are activators: sulfonate and azide. Still some derivatives have only slight or no effect on angiogenesis: carboxylate 7 and amine 6. Carboxylate 10 exhibited the same effect as acidic ion-exchange resin (Amberlyst 15-H^+) then led to the removal of the monosulfate and isopropylidene groups. In the case of nitrile 5, sodium cyanide was used in N,N-dimethylformamide without adding a base in order to avoid a premature hydrolysis of the functional group. Starting from nitrile 5, hydrolysis or hydrogenolysis led to carboxylate 7 or aminomethyl derivative 6, respectively. Nucleophilic substitution of the cyclic sulfate is, therefore, a versatile method that gives mannose derivatives in good to excellent (quantitative) yields. Obtaining agents for biological evaluation in such a flexible manner allowed us to unveil the best candidates for controlling angiogenesis.

### Table 1. Cytotoxicity, tumor growth and evaluation of angiogenic effects of M6P analogues

<table>
<thead>
<tr>
<th>Compd</th>
<th>ARA[b,c] no. sprouts [%]</th>
<th>ARS[d] %</th>
<th>Cell toxicity[e] %</th>
<th>Vol[c] [mm^3]</th>
<th>B16 Tumor growth survival [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sunitinib</td>
<td>21 ± 13</td>
<td>49 ± 2</td>
<td>78 ± 5.5</td>
<td>123 ± 8.5</td>
<td>107 ± 2.5</td>
</tr>
<tr>
<td>11</td>
<td>34 ± 29</td>
<td>43 ± 2</td>
<td>110 ± 3.5</td>
<td>109 ± 4.9</td>
<td>101 ± 3</td>
</tr>
<tr>
<td>9</td>
<td>97 ± 9</td>
<td>69 ± 3.5</td>
<td>80 ± 5.5</td>
<td>99 ± 3</td>
<td>101 ± 2</td>
</tr>
<tr>
<td>8</td>
<td>45 ± 21</td>
<td>70 ± 0.5</td>
<td>113 ± 4</td>
<td>119 ± 3.5</td>
<td>103 ± 4.5</td>
</tr>
<tr>
<td>10</td>
<td>N.D.</td>
<td>73 ± 0.5</td>
<td>110 ± 2.5</td>
<td>110 ± 1.9</td>
<td>104 ± 1.5</td>
</tr>
<tr>
<td>6</td>
<td>58 ± 33</td>
<td>81 ± 2</td>
<td>80 ± 10</td>
<td>101 ± 13.5</td>
<td>120 ± 23</td>
</tr>
<tr>
<td>PBS control</td>
<td>88 ± 21</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>2.45 ± 0.97</td>
</tr>
<tr>
<td>7</td>
<td>71</td>
<td>123 ± 7.5</td>
<td>119 ± 2.5</td>
<td>110 ± 2</td>
<td>104 ± 1.5</td>
</tr>
<tr>
<td>4</td>
<td>115 ± 7</td>
<td>125 ± 3.5</td>
<td>84 ± 9</td>
<td>90 ± 5.5</td>
<td>121 ± 5</td>
</tr>
<tr>
<td>3</td>
<td>56</td>
<td>130 ± 3.5</td>
<td>131 ± 7.5</td>
<td>86 ± 4.5</td>
<td>98 ± 8.5</td>
</tr>
<tr>
<td>ECGS</td>
<td>N.A.</td>
<td>172 ± 8.5</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

[a] N.A.: not applicable; N.D.: not done; [b] Aortic ring assay (ARA); [c] data represent the mean ± SD; data were analyzed by one-way ANOVA or two-way ANOVA for repeated measures when required. [d] Angiogenic relative surface (ARS) was determined using a chorioallantoic membrane (CAM) assay; data represent the mean ± SD versus the PBS control. [e] Data represent the mean ± SD versus the control. [f] Tumor volume was determined on day 19. Animal experiments complied with the European and French laws and with the guiding principles for experimental procedures as set forth in the declaration of Helsinki.
MeM6P (11) itself, with the number and the length of sprouts being equivalent.

The second biological assay used to evaluate our M6P derivatives was the avian chorioallantoic membrane (CAM) assay. Control- or M6P-analogue-treated membranes were deposited on nascent CAM at embryonic day 7 and grown for 4 days in ovo at 38°C. The same positive and negative controls were included as before. Quantification of the angiogenic response was carried out by measuring the area of neovascularization on each particular membrane. These experiments demonstrate divergent activities of the synthesized compounds (Figure 1).

Figure 1. Chorioallantoic membrane (CAM) assays performed with mannose-6-phosphate (M6P) analogues 3, 4, 6–11, the antiangiogenesis inhibitor sunitinib and the angiogenesis activator, endothelial cell growth supplement (ECGS). Also shown is the phosphate-buffered saline (PBS) control experiment for comparison. The values given represent the extent of angiogenesis, where the PBS control is defined as 100%.

As before, some M6P derivatives were identified as CAM inhibitors: sulfonate 9, malonate 8, amine 6 and MeM6P 11, while other derivatives behaved as CAM activators: phosphonate 3, azide 4 and carboxylate 7. In the inhibitor group, compared to the control, we observed 43% of neovascular vessels for phosphonate 11, 69% for sulfonate 9, 81% for amine 6, 70% for malonate 8, and 73% for carboxylate 10. In the activator group, compared to the control, an increase in neovascular vessels of 123% was observed for azide 7, 125% for carboxylate 4, and 130% for phosphonate 3. It is worth pointing out that the azide and carboxylate derivatives, both of which displayed lower activities compared with the other test compounds, are not bioisosteric analogues of M6P. It is also noteworthy that the results of our two assays do not correspond exactly. During our study, we analyzed two different angiogenic processes, sprouting using aortic ring assay and intussusception (splitting) angiogenesis with CAM assays. These two processes can be differently modulated by M6P analogues. This is not unusual in the angiogenesis arena and explains why multiple assays are often needed to portray the efficacy of a potential drug.

Finally, compounds showing the most potent angiogenic properties, namely MeM6P (11) for activation and phosphonate 3 for activation, as well as the azido 4 (moderate activity), were tested in a B16 melanoma tumor growth model and their cytotoxicity was also evaluated in primary human endothelial cell cultures. Indeed, the antiangiogenic properties of some compounds could be the result of specific cell toxicity. At the three concentrations tested, slight or no effect on the cell number in primary human endothelial cell cultures was observed after 48 h exposure (Table 1). Mice were injected with B16F1 cells (day 0). The mice were then divided into groups and were treated (i.p.) with 300 mg kg⁻¹ of test compound three times a week, starting from day 0. MeM6P (11) showed 79% tumor growth inhibition and 75% survival at day 19. Azide 4 is also an inhibitor of tumor growth but to a lesser extent (50%). No effect was observed for phosphonate 3 (Table 1).

In conclusion, we observed that, of the M6P analogues evaluated, some display proangiogenic activities, while others display antiangiogenic activities. These latter analogues were tested in a melanoma B16 tumor growth model, and at least two of them have been shown to inhibit tumor growth. We have clearly demonstrated that M6P and its analogues assist in the control of neoangiogenesis, and these compounds can be considered as leads for the development of a novel class of therapeutics.

We have presented an efficient method for synthesizing M6P analogues. This route can be used to develop additional carbohydrate analogues modified at the C-6 position, thereby allowing access to a large variety of original carbohydrate mimics. We also investigated the function of these monocarbohydrates during angiogenic processes, showing for the first time that monocarbohydrates possess angiogenic activities via the M6PR with no apparent toxicity. These results open the possibility for developing angiogenesis regulator carbohydrates as anticancer agents (inhibitors) or for the treatment of cardiovascular disease (activators). It is clear that our preliminary results are a promising start in the use of carbohydrates as angiogenic regulators. The in vitro, in ovo, and in vivo assays have divided the M6P analogues tested into angiogenesis activators and inhibitors. The mechanism of action of M6P analogues as angiogenesis regulators has still to be elucidated.
but it seems obvious that the M6P receptor is involved. Investigation into the mechanism of M6P analogue action is underway now that a family of regulators is in hand.

### Experimental Section

Supporting Information: Experimental protocols for the synthesis and biological evaluation of the analogues described here can be found on the WWW under http://dx.doi.org/10.1002/cmdc.201100293.

**Abbreviations:** para-Toluenesulfonic acid (p-TsOH); 2,2-dimethoxy-propanes (DMP); 4-dimethylaminopyridine (DMAP); 4-dimethylamino-pyrrolidine (DMAP); N,N-dimethylformamide (DMF); hexamethylphosphorous triamide (HMPT); tetrahydrofuran (THF); trimethylsilyl bromide (TMSBr).

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