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A focused sulfated glycoconjugate Ugi library for probing heparan sulfate-binding angiogenic growth factors

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ABSTRACT

A library of small molecule heparan sulfate (HS) mimetics was synthesized by employing the Ugi four-component condensation of D-mannopyranoside-derived isocyanides with formaldehyde as the carbonyl component and a selection of carboxylic acids and amines, followed by sulfonation. The library was used to probe the subtle differences surrounding the ionic binding sites of three HS-binding angiogenic growth factors (FGF-1, FGF-2 and VEGF). Each compound features 3 or 4 sulfo groups which serve to anchor the ligand to the HS-binding site of the protein, with a diverse array of functionality in place extending from C-1 or C-6 to probe for adjacent favorable binding interactions. Selectivity of binding to these proteins was clearly observed and supported by molecular docking calculations.

Finding more efficient approaches to accelerate lead discovery and optimization has been a continuing theme for drug discovery in recent years. Out of many outstanding strategies, multi-component reactions have attracted significant attention from both the academic and industrial sectors due to their efficiency in the generation of chemical diversity.[1] The Ugi four-component condensation is the most important isocyanide-mediated multi-component reaction, and has been explored in many drug discovery and development applications[2] including in the glycomics area.[3] Some years ago the potential of the Ugi reaction for targeting angiogenic heparan sulfate (HS)-binding growth factors such as fibroblast growth factors 1 and 2 (FGF-1 and -2) and vascular endothelial growth factor (VEGF) was demonstrated.[4,5] Herein we report a focused library approach utilizing the Ugi reaction for probing the ligand–protein binding sites in order to identify higher affinity small molecule leads for these HS-binding growth factors.

The FGFs and VEGF are important mediators of angiogenesis and thus are attractive targets for drug discovery.[6] They initiate cell signaling cascades that lead to angiogenesis by initial formation of ternary complexes with HS and their cognate receptors. Inhibiting angiogenesis by targeting the HS-binding site with HS mimetics and thus blocking the formation of these ternary complexes is a viable therapeutic strategy for cancer.[7] The FGFs have been widely studied and a number of X-ray crystal structures are available, including in complex with heparin-derived oligosaccharides,[8] and in ternary complex with its receptor (FGFR) and a heparin-derived oligosaccharide.[9] The HS-binding sites of these proteins share a common feature, that is, a densely charged, cationic, shallow groove.

Over the past decade, we have explored the use of small molecule HS mimetics as potential inhibitors of FGF- and/or VEGF-mediated angiogenesis for the development of novel cancer therapeutics.[10] Such approaches avoid the inherent synthetic complexity associated with natural HS oligosaccharide sequences and have the potential for modifying biological activity by simple chemical modifications. Among the various approaches, we reported[10e] the use of a panel of linked sulfated cyclitols containing the core structure **3** (Fig. 1) to probe various HS-binding proteins. These studies indicated that ligand selectivity towards such proteins could be influenced by subtle changes in the size and orientation of the spacers between the two highly charged centres, which is in agreement with the generally accepted view that different domains or structural motifs of HS bind to different proteins to mediate their effects.[11]

In the current design strategy a simple ionic binding motif on a monosaccharide scaffold that could act to 'anchor' the ligand to the HS-binding site on the target protein was envisaged. The Ugi reaction would then be utilized to decorate the scaffold, via various linkers, with a diverse range of functional groups in order to probe for additional favorable binding interactions. This concept allows for the rapid assembly of a library of small molecules for use as a tool to map the structural tolerances on the surrounding surface of the ionic binding site of these related proteins, with the aim of

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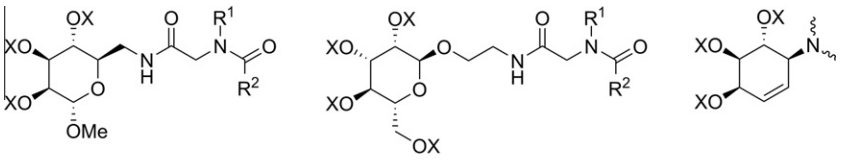


Figure 1. Library design based on Man-6-isocyanide (**1**; series 1) and Man-1-isocyanide (**2**; series 2) and the core structure motif of linked cyclitols (**3**). X = SO_3Na.

improving affinity and selectivity for each individual protein. This approach also seeks to reduce the overall charge of the ligands by minimizing the number of sulfo groups in the HS-binding anchor and by introducing mostly hydrophobic (aliphatic or aromatic) groups via the Ugi reaction.

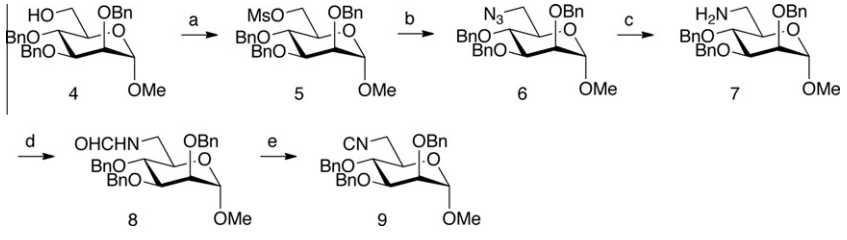
To limit the number of possible structures, we selected only two monosaccharide scaffolds **1** and **2** based on D-mannopyranose with extension from the more readily accessible positions C-1 and C-6 (Fig. 1). These common scaffolds were designed to contain the isocyanide component for the Ugi reaction whilst the carboxylic acid and amine components could be selected from a vast range of commercially available structures. The simplest carbonyl component, formaldehyde, was chosen as the fourth partner to avoid the complexity of generating an additional diastereomeric centre. The selection of D-mannose was also based on existing internal programs involving this sugar as well as the presence of a pair of *cis*-oriented vicinal hydroxyl groups which can be sulfonated, a feature common to the linked cyclitol core structure **3**.

The preparation of mannoside-6-isocyanide **9** is outlined in Scheme 1. By following standard functional group transformations, the alcohol **4**[12] was converted into the corresponding amine **7** in 54% overall yield. Subsequent formylation and dehydration afforded **9** in high yield (93%). The corresponding C1-isocyanide **10** was prepared as reported earlier.[4] The structures of the intermediates and products were characterized by ^1H and/or ^{13}C NMR spectroscopy.[13] Both building blocks **9** and **10** showed characteristic signals for isocyanide carbon at 157.63 and 157.57 ppm, respectively, in ^{13}C NMR spectra.

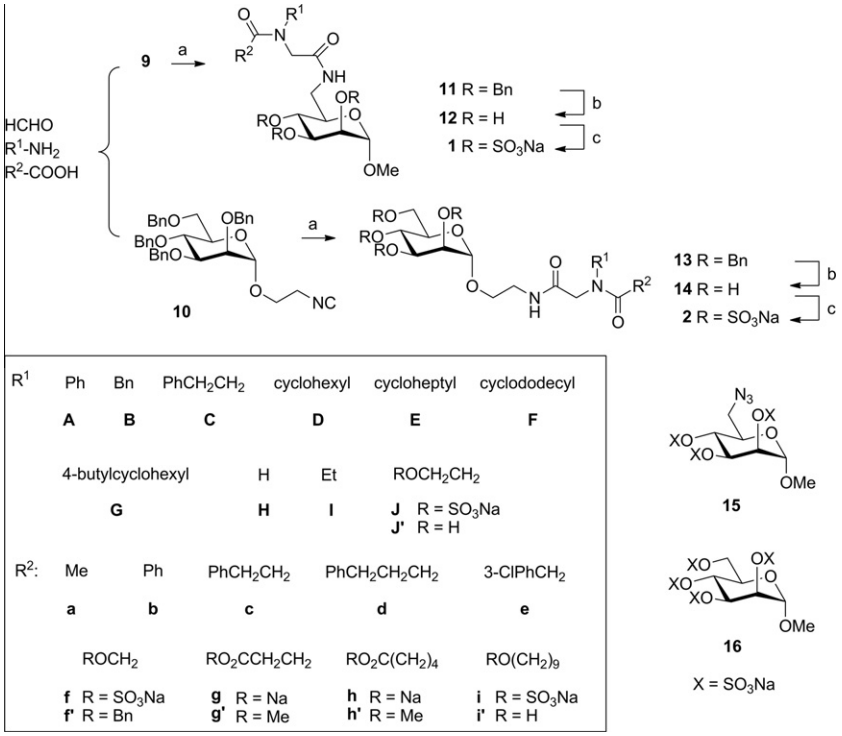
The components for the Ugi library construction are illustrated in Scheme 2. Although formaldehyde has not been used frequently in the Ugi reaction, we found in general that the yields using this carbonyl source were good. In a few cases, elevated temperatures were required to increase the yield (such as entry 9, Table 1). The presence of free hydroxyl groups was generally well tolerated during the Ugi reaction except in the case of glycolic acid, which gave an unidentified mixture. However, protection of the hydroxyl group as the benzyl ether gave the desired Ugi adduct in good yield (69%, ***entry 13, Table 1). Global debenzylation was carried out in quantitative yield by hydrogenolysis with 20% $Pd(OH)_2$ on charcoal as catalyst. The crude polyol, after drying under high vacuum in the presence of phosphorus pentoxide, was used directly for the sulfonation step. Purification of the multi-charged products was not always straightforward and sometimes required a combination of several purification techniques, such as silica gel or size-exclusion column chromatography and solid phase extraction (SPE). For those compounds with an extra carboxylate group, which was introduced as the methyl ester during the Ugi reaction step, the crude sulfated product was further subjected to a sodium hydroxide mediated hydrolysis step prior to the standard purification. The purified product fractions were individually analysed by capillary electrophoresis (CE), pooled and lyophilized to give the final sulfated material as an amorphous powder (purity ⩾ 98% by CE).

The binding affinities of the library for FGF-(1,2) and VEGF were measured using a surface plasmon resonance (SPR) solution affinity assay (Table 1).[14] The affinities for the non-Ugi elaborated parent compounds[10f,15] trisulfate **15** and tetrasulfate **16** are also provided for comparison. The measured K_d values clearly showed the varied preference of each protein towards the ligands, with the affinities spanning 38-, 7- and 104-fold differences for FGF-1, FGF-2 and VEGF, respectively. For FGF-1, either an aromatic group or an extra negative charge is preferred to an aliphatic group for increased affinity. Additional charged groups either in the D-mannoside ring or as an attached functional group did not always correlate with improved affinity. For example, **1Aa**-**1Ba**-**1Ca**-**1Ig**-**1Ih**-**1Ii** showed lower affinities compared with their corresponding series 2 compounds. **1He** (37 μM) not only binds more tightly than its Series 2 analogue **2He** (171 μM) but is also one of the best compounds out of the panel for FGF-1. Although extra charged groups (motifs g, h, i) had a greater influence when delivered in Series 2 (20–44 μM) compared with Series 1 (111–338 μM), it is aromatic groups that result in the tightest binding compound (**2Ca** at 11.8 μM) and provide more useful SAR information (the homologues **2Ba** and **2Aa** at 24.8 and 97.4 μM, respectively). The affinities of the ligands towards FGF-2 showed the smallest variation, with the best two containing either a flexible aromatic group (113 μM for **2Ca**) or an extra negative charge (118 μM for **2Ig**).

For VEGF, ligand binding affinities followed a similar trend to FGF-1, which may imply that there is some similarity in their HS-binding domains. However, one striking difference was the intolerance of VEGF to cyclic aliphatic rings (1–3 mM for D, E, F, G), while an aromatic group (28.8–109 μM for motif A, B, C, d, e)



Scheme 1. Reagents and conditions: (a) MsCl (1.5 equiv), DMAP (0.11 equiv), Et_3N (3 equiv), DCM (0.06 M), 0–18 °C, 18 h, 100%; (b) NaN_3 (5 equiv), DMF (0.2 M), 90 °C, 3 h, 63%; (c) (i) PPh_3 (1.3 equiv), THF (0.2 M), 18 °C, 1 h, (ii) H_2O (20 equiv), 18 °C, 18 h, 85%; (d) Ac_2O (30 equiv), HCO_2H (0.06 M), 18 °C, 3.5 h; (e) $POCl_3$ (2 equiv), TEA (4 equiv), DCM (0.05 M), 0 °C, 2 h, 93% (two steps).



Scheme 2. Reagents and conditions: (a) HCHO (1 equiv, 36.5–38.0% w/w, aqueous solution stabilized with 10% MeOH), R^1NH_2 (1 equiv), R^2CO_2H (1 equiv), MeOH (0.4 M), 18 or 60 °C, 24 h, 36–75%; (b) 20% $Pd(OH)_2$ on charcoal, MeOH, 50 PSI, 2 h, 100%; (c) (i) $SO_3{\cdot}Me_3N$ or $SO_3{\cdot}$pyridine complex (3 equiv per hydroxyl), DMF (0.04 M), 60 °C, 18 h, (ii) pH 9, 1 M NaOH, 0 °C, evaporated to dryness, (iii) only for series with methyl ester (g′ and h′), 1 M NaOH, 18 °C, 18 h, (iv) Bio-Gel P-2 (0.1 M NH_4HCO_3) and/or LH-20 (DI water) column chromatography, silica gel chromatography, or SPE with Strata-X cartridge or Strata-NH_2 cartridge, (v) ion-exchange resin column (AG®-50W-X8, Na^+ form), (vi) freeze–dry (R^1 and R^2 see Table 1).

Table 1
Yield of the Ugi library production and dissociation constants measured for sulfated products binding to FGF-(1,2), VEGF

Entry	Yield (%)[a]		Structural Code	K_d (μM)[b]		
	Ugi reaction (step a)	Sulfated product (steps b,c)		FGF-1	FGF-2	VEGF
1	64	45	**1Aa**	196 ± 29	480 ± 70	76[#]
2	38	40	**1Ba**	140 ± 12	192 ± 16	77[#]
3	42	32	**1Ca**	224 ± 30	680 ± 60	109[#]
4	60	51	**1Da**	310 ± 50	413 ± 8	1700[#]
5	82	45	**1Ea**	370 ± 40	410 ± 70	3000[#]
6	62	24	**1Fa**	265 ± 28	480 ± 90	1200[#]
7	49	15	**1Ga**	243 ± 21	540 ± 30	1000[#]
8	56	25	**1He**	37 ± 10	430 ± 100	45.3 ± 1.1
9	56[c,d]	63	**1Ja**	95 ± 19	240 ± 50	280 ± 210
13	69[e]	67	**1If**	174[#]	ND	512.0 ± 2.8
10	65[f]	18	**1Ig**	338 ± 18	291 ± 16	207[#]
11	66[f]	52	**1Ih**	160 ± 60	480 ± 120	104[#]
12	52[d]	73	**1Ii**	111 ± 26	240 ± 80	545[#]
14	76, 97[d]	46	**1Ib**	366 ± 9	480 ± 80	1100[#]
15	82	29	**1Ic**	ND	ND	596[#]
16	67	41	**1Id**	500 ± 400	400 ± 110	81[#]
17	24, 74[d]	68	**2Aa**	97 ± 19	240 ± 40	402[#]
18	67	14	**2Ba**	25 ± 16	287 ± 27	76.6[#]
19	12, 45[d]	57	**2Ca**	11.8 ± 2.1	113 ± 20	29[#]
20	37	28	**2He**	170 ± 50	837[#]	91[#]
21	65[f]	28	**2Ig**	43 ± 14	122 ± 5	40[#]
22	75[f]	51	**2Ih**	44 ± 13	188 ± 18	81[#]
23	51[d]	34	**2Ii**	20 ± 6	157 ± 25	50[#]
24	—	—	**15**	343 ± 19	210 ± 15	630 ± 100
25	—	—	**16**	130 ± 50	340 ± 150	198[#]

[a] Unoptimized percentage yields. In the cases of low yield for final sulfated product, the reason was the generation of undersulfated by-products, which required tedious purification by chromatography and/or SPE. Experimental details for the Ugi and sulfonation reactions have been reported previously.[4]
[b] The average and SD of at least two measurements. [#]Data from a single measurement only. ND: no data available.
[c] 60 °C, 18 h (normal Ugi conditions: rt 18 h).
[d] Sulfate (OSO_3Na) in final product was introduced at this step as hydroxyl (OH).
[e] Sulfate (OSO_3Na) in final product was introduced at this step as benzyl ether (OBn).
[f] Carboxylate (CO_2Na) in final product was introduced at this step as methyl ester (CO_2Me).

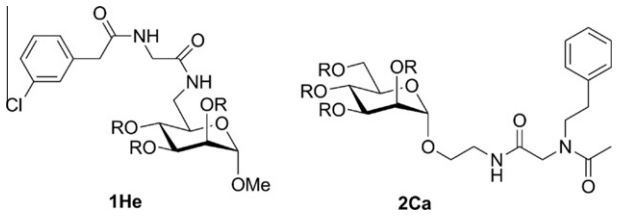


Figure 2. Structures of the most potent compounds from each series. R = SO_3Na.

was highly active with a negatively charged functional group ranked next (40–283 μM for J, f, g, h, i). It is interesting to note there are exceptions when an aromatic group or a negative charge is delivered at less than ideal positions such as in **1Ib**, **1Ic** and **2Aa** or **1Ii** (402–1100 μM). As the linker group between the sulfated D-mannoside ring and the probing functional group is varied from Series 1 and 2 (5–6 atoms vs 7–8 atoms), VEGF displays a large spatial tolerance for aromatics, for example, similar activities (45–109 μM) among **1Aa**-**1Ba**-**1Ca**-**1He** and **2Ba**-**2Ca**-**2He**). An extra negative charge clearly improves the affinity, as it does in the D-mannoside ring of Series 2. However, it is not always the case that increased charge in the molecule results in higher affinity, with the best lead out of either series bearing an aromatic motif (**1He** at 45.3 μM and **2Ca** at 29 μM, Fig. 2).

We have recently reported the synthesis of an alternative library of HS mimetics based on the D-mannopyranose scaffold in which diversity was introduced at C6 via click chemistry (Cu^I-catalyzed Huisgen azide–alkyne cycloaddition reaction) or a click-Swern oxidation–Wittig reaction sequence.[10f] Members of the earlier library in general had poorer affinities for FGF-1, VEGF and especially FGF-2 compared with the current library, most likely as a consequence of possessing one less sulfate group per molecule (an allyl group is generally present at C4). The additional flexibility of the Ugi-derived side chains would also appear to contribute significantly to the improved affinity (see below).

Previous molecular docking studies on FGF-1 and FGF-2 with small molecule HS-mimetics have shown that the predicted locations of bound sulfo groups display good overlap with the positions observed for sulfo groups from co-crystallized heparin-derived oligosaccharides.[10c,15] Molecular docking calculations were thus performed to examine the binding modes of compound **2Ca** with FGF-1 and FGF-2 (Fig. 3). Docking studies were undertaken with Molegro Virtual Docker (MVD) using the MolDock SE algorithm.[16] The ligand search space was confined to an 11 Å sphere originating from the ligand binding sites of FGF-1 (PDB code 2AXM) and FGF-2 (PDB code 1BFB) based on the corresponding ligand in the crystal structure. The location of the sulfate groups of the bound ligands in the crystal structures of these complexes were used as a template to score higher for poses that resemble this binding mode. Ligands and water molecules were omitted from the coordinate files before docking. In the docking of the complex between **2Ca** and FGF-1, the C6 sulfate group in the inhibitor forms hydrogen bonds with the nitrogen atom (3.0 Å) in the side-chain amide of Gln 127 and with the ε-amino group of Lys 118 (3.1 Å). The latter nitrogen atom also forms a hydrogen bond (2.9 Å) with the C2 sulfate group. The other two oxygen atoms in the C2 sulfate form hydrogen bonds with the protein; (i) between the backbone amide of Lys 113 (2.7 Å) and (ii) between the nitrogen of the side-chain amide of Asn 18 (2.8 Å). A third sulfate group (C4) forms a hydrogen bond to the backbone amide of Lys 128 (2.7 Å). The remainder of the inhibitor forms van der Waals contacts with the side-chain of Arg 122 and the aromatic ring forms π–cation interactions with the side-chain guanidino group of this residue.

In the docking calculations for the complex between **2Ca** and FGF-2, the C2 sulfate group forms two hydrogen bonds (both

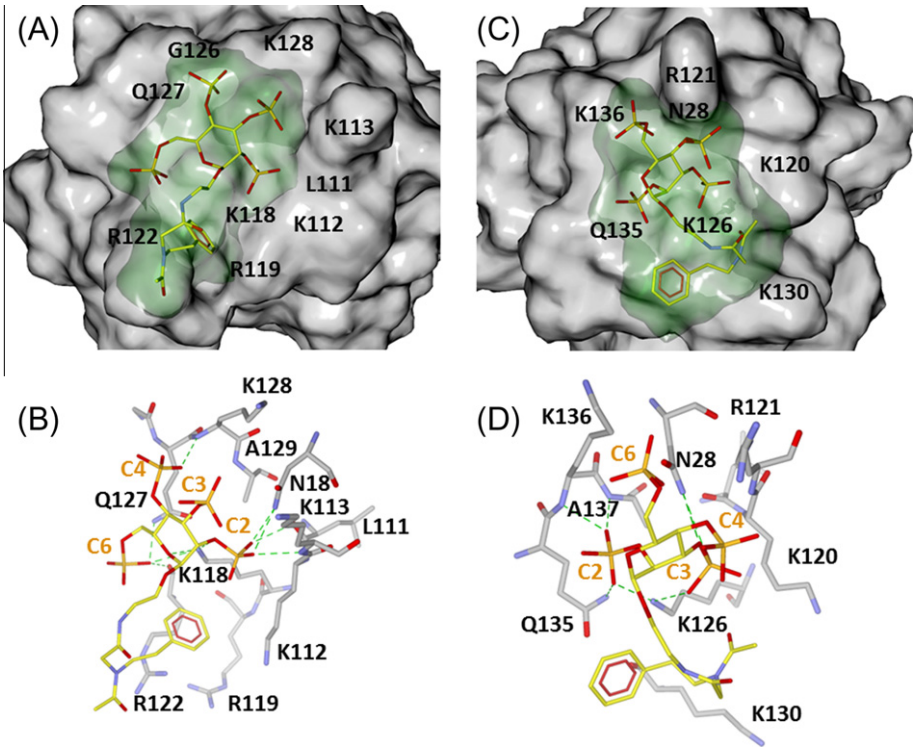


Figure 3. Docking of compound **2Ca** to FGF-1 (A, B) and FGF-2 (C, D). Both docking calculations show that the inhibitor can bind to the FGFs primarily through its sulfate groups, forming hydrogen bonds and electrostatic interactions between the negatively charged sulfate oxygen atoms and the conserved residues (Lys 118/126, Gln 127/135, Ala 129/137, Asn 18/28). The side chain of the inhibitor forms van der Waals interactions with both FGFs. The molecular surface of the protein (top) is shown in grey and inhibitor surface is shown in transparent green.

2.6 Å) with the nitrogen atoms in the backbone amide of residues Ala 137 and Lys 136. The C2 and the C3 sulfates also interact with the positively charged ε-amino group of Lys 126 (2.7 Å), and the C3 sulfate group additionally forms a hydrogen bond with a nitrogen atom (3.0 Å) in the side-chain amide of Asn 28. The side-chain guanidino group of Arg 121 is wedged between the other two sulfate groups (C4 and C6) forming ionic interactions (3.0–3.2 Å). The side chain of **2Ca** binds to FGF-2 through van der Waals interactions with the side-chains of residues Lys 130 and Gln 135.

In conclusion, by harnessing the powerful Ugi four component condensation, a library of HS mimetics was quickly assembled and used to probe the binding environment of some angiogenic HS-binding growth factors. The affinities measured for these monosaccharide derivatives are close to those generally observed for polysulfated di- to tetrasaccharides,[10a,c] especially those leads bearing fewer charged groups. A clear trend for protein preference, particularly an aromatic group for FGF-1 and VEGF, was observed. These observations are generally in agreement with previous studies on modified heparin polysaccharides which showed that increasing lipophilic modifications can improve affinity for VEGF and FGF-1 but not FGF-2.[17] Future studies should focus on optimizing the sulfation pattern around the monosaccharide motif as well as investigating a combination of more than one hydrophobic group to maximize non-ionic binding contributions with an expectation to achieve higher affinity small molecule ligands.

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Supplementary data

Supplementary data (copies of NMR spectra for intermediates and final compounds. Experimental details and characterization data for selected compounds) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.08.001.

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13. *^{1}H and ^{13}C NMR data for compound* **9**: ^{1}H NMR (400 MHz, $CDCl_3$, 7.27): 7.42-7.29 (m, 15 H, 3 × Ph), 5.01, 4.61 (ABq, J_{AB} = 11.2 Hz, PhCH_2), 4.78, 4.72 (ABq, J_{AB} = 12.0, PhCH_2), 4.77 (d, 1 H, $J_{1,2}$ = 1.6, H1), 4.65, 4.63 (ABq, J_{AB} = 11.6, PhCH_2), 3.93 (dd, 1H, $J_{3,4}$ = 9.2, $J_{2,3}$ = 2.8, H3), 3.85 (dd, 1 H, $J_{4,5}$ = 9.6, H4), 3.83 (dd, 1 H, H2), 3.73 (ddd, 1H, $J_{5,6a}$ = 7.2, $J_{5,6b}$ = 2.0, H5), 3.69 (dd, 1 H, $J_{6a,6b}$ = 15.2, H6b), 3.55 (dd, 1 H, H6a), 3.37 (s, 3H, CH_3O). ^{13}C (100 MHz, $CDCl_3$, 77.0): 157.57 (NC), 138.00, 137.94, 137.83, 128.47, 128.362, 128.355, 128.34, 127.98, 127.89, 127.72, 127.68, 127.65, 127.57, 98.99 (C1), 79.90, 75.17, 74.96, 74.15, 72.72, 71.89, 69.60, 54.93, 43.10.
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