60 s. For graduated PCR, 50 pmol of each primer and 2.5 units of Taq DNA polymerase in PCR buffer to a total volume of 50 μl were processed for 25 cycles at 94°C for 15 s and at 40°C for 60 s.

6. All gels were 3 or 5% agarose (NuSieve, FMC BioProducts, Rockland, ME) in tris-borate-EDTA buffer with ethidium bromide staining (*14*).
7. Oligonucleotides were 5′ biotinylated with LC Biotin-ON Phosphoramidite (Clontech). To obtain single-stranded DNA, the product from Step 3 was amplified by PCR with the use of primers O_0 and biotinylated $\overline{O}_6$. The amplified product was annealed to streptavidin paramagnetic particles (Promega, Madison, WI) by incubating in 100 μl of 0.5× saline sodium citrate (SSC) for 45 min at room temperature with constant shaking. Particles were washed three times in 200 μl of 0.5× SSC and then heated to 80°C in 100 μl of ddH_2O for 5 min to denature the bound double-stranded DNA. The aqueous phase with single-stranded DNA was retained. For affinity purification, 1 nmol of biotinylated $\overline{O}_1$ was annealed to particles as above and washed three times in 400 μl of 0.5× SSC. Single-stranded DNA was then incubated with these particles in 150 μl of 0.5× SSC for 45 min at room temperature with constant shaking. Particles were washed four times in 400 μl of 0.5× SSC to remove unbound single-stranded DNA and then heated to 80°C in 100 μl of ddH_2O for 5 min to release single-stranded DNA bound to $\overline{O}_1$. The aqueous phase with single-stranded DNA was retained. This process was then repeated for $\overline{O}_2$, $\overline{O}_3$, $\overline{O}_4$, and $\overline{O}_5$.
8. From a graph theoretic point of view, the use of equal quantities of each oligonucleotide in the ligation reaction is not optimal and leads to the formation of large amounts of molecules encoding paths that do not start at vertex 0 nor end at vertex 6. A better way to proceed is to first calculate a flow on the graph and to use the results to determine the quantity of each oligonucleotide that is necessary.
9. On an n vertex graph G with designated vertices v_{in} and v_{out}, there may be multiple Hamiltonian paths. If it is desirable to have an explicit description of some Hamiltonian path, that can be accomplished by extending the algorithm as follows. At the end of Step 4, one has a solution (in the chemistry sense) containing molecules encoding all Hamiltonian paths for $\langle G, v_{in}, v_{out}\rangle$. The graduated PCR performed at the end of Step 4 will produce the superimposition of the bands corresponding to all of these Hamiltonian paths in the $n - 1$ successive lanes. For some lane i, a band of least weight (40 bp) will appear. This indicates that some Hamiltonian path begins with v_{in} and proceeds directly to vertex i. By amplifying by PCR the solution with primers O_i and $\overline{O}_n$, running a gel, and excising the $20 \times (n - 1)$ bp band, one can ensure that only those molecules encoding such Hamiltonian paths will be retained. One now has a solution containing molecules encoding all Hamiltonian paths for $\langle G', i, v_{out}\rangle$, where G' is the graph where vertex v_{in} has been removed. This procedure is now iterated.
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Efficient Neutralization of Primary Isolates of HIV-1 by a Recombinant Human Monoclonal Antibody

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The ability of antibodies to neutralize diverse primary isolates of human immunodeficiency virus–type 1 in vitro has been questioned, with implications for the likely efficacy of vaccines. A recombinant human antibody to envelope glycoprotein gp120 was generated and used to show that primary isolates are not refractory to antibody neutralization. The recombinant antibody neutralized more than 75 percent of the primary isolates tested at concentrations that could be achieved by passive immunization, for example, to interrupt maternal-fetal transmission of virus. The broad specificity and efficacy of the antibody implies the conservation of a structural feature on gp120, which could be important in vaccine design.

Protection from viral disease has traditionally been associated with the preexistence in serum of antibodies capable of neutralizing virus in vitro. Indeed, vaccines are frequently assessed on the ability to elicit neutralizing antibody responses. In the case of human immunodeficiency virus–type 1 (HIV-1), there was initial optimism about the likely efficacy of subunit vaccines given that vaccinee sera from several trials were capable of neutralizing laboratory isolates of virus in vitro (*1*, *2*). The grounds for optimism were shaken when it was found that the vaccinee sera were largely ineffective against primary isolates of HIV-1 (*2*). Some discussion subsequently centered around the validity of standard HIV-1 neutralization assays when applied to primary isolates (*2*–*5*). If the assays are meaningful, then they call into question the ability of antibody to effectively neutralize a spectrum of primary isolates. Hyperimmune pooled human plasma preparations are capable of neutralizing a number of primary isolates (*3*–*5*), but they represent a combination of specificities that might be difficult to elicit by all except the most complex vaccines (*6*). A single antibody able to effectively neutralize a broad spectrum of primary isolates would validate the vaccine approach and would provide a template for vaccine design. Furthermore, it would constitute a reagent for passive immunotherapy such as in the interruption of maternal-fetal transmission. We describe here such a human antibody derived by recombinant methods (*7*).

The generation of the antibody Fab fragment b12 from a combinatorial phage display library has been described previously (*8*). Fab b12 is directed to the CD4 binding site of gp120 and is a potent neutralizer of the HIV-1 laboratory strains IIIB and MN (*9*–*11*). Selection for potency and strain cross-reactivity was achieved through experimental design. The library donor was a long-term asymptomatic U.S. male, presumably infected with a clade B strain of HIV-1; the antigen for affinity selection was gp120 from the atypical IIIB strain, thereby favoring selection of cross-reactive antibodies. A large number of bacterial supernates containing antibody Fab fragments to gp120 (anti-gp120) at low initial concentrations were directly screened for neutralizing ability to find the most potent Fabs. Although Fab b12 is capable of neutralizing some primary isolates (*12*), the corresponding whole antibody molecule is likely to be more effective. Therefore, Fab b12 was converted to a whole immunoglobulin G1 (IgG1) molecule by cloning the variable region of Ig heavy chain (V_H) and light chain genes into a vector created for high-level mammalian expression (*13*). The whole antibody IgG1

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b12 was expressed in Chinese hamster ovary (CHO) cells and purified by affinity chromatography.

IgG1 b12 was initially tested against the laboratory strains MN and IIIB in two neutralization assays in laboratories that recently tested a panel of monoclonal antibodies as part of the National Institute of Allergy and Infectious Diseases–World Health Organization (NIAID-WHO) Antibody Serological Project (*14*). IgG1 b12 showed 50% neutralization titers of 3 ng/ml for the MN strain and 7 ng/ml for the IIIB strain when plaque formation (*15*) was used to indicate antibody inhibition of infectivity (Table 1), and titers of 20 ng/ml for both MN and IIIB strains when syncytial formation was used as the reporter assay (*16*). These titers suggest that the antibody is approximately two orders of magnitude more potent than other CD4 site antibodies used in the Project and comparable to the best antibodies directed to the V3 loop. However, whereas the latter are strongly strain specific, IgG1 b12 is roughly equally effective against MN and IIIB. The antibody is comparable in potency to a CD4-IgG molecule in these assays. In a separate assay in which p24 production was the measure of infectivity (*17*), 50% neutralization titers of less than 40 ng/ml were found for both MN and IIIB strains (Fig. 1).

IgG1 b12 was next tested against a set of 10 primary virus isolates in the p24 reporter assay (*17*). The viruses were isolated from individuals from various locations in the United States and with varying disease status (Fig. 1). The viruses had been cultured only once or twice in peripheral blood mononuclear cells (PBMCs). Viral stocks were grown in PBMCs and the assay was carried out with these cells. IgG1 b12 essentially completely neutralized 7 of 10 isolates at 5 μg/ml (Fig. 1), and all the isolates were 50% neutralized at ≤1 μg/ml.

The ability of IgG1 b12 to neutralize an additional set of 14 primary isolates was then examined in a microplaque assay (*15*). The set was chosen to contain a high proportion of isolates that were relatively refractory to antibody neutralization by sera from other HIV-1–infected individuals (*5*). Viruses were grown in PBMCs and the assay carried out in MT2 cells. This step limits study to viruses that grow in this cell line but provides an alternative measure of neutralization. Table 1 shows that 10 of 14 primary isolates were neutralized with 50% titers generally higher than those in the p24 assay (Fig. 1). Four isolates, which were not neutralized even by a 1:10 dilution of pooled human plasma, were neutralized by IgG1 b12. Most of the viruses reported in Table 1 were isolated from U.S. donors although two, both of which are neutralized by IgG1 b12, were from Ugandan donors and were assigned to clade D.

One of the most likely roles for passive immunotherapy with antibody is in the interruption of maternal-fetal transmission of virus, as supported by recent reports (*18*) which suggest that transmission correlates with an absence of maternal neutralizing antibody to the transmitted virus. Therefore, the ability of IgG1 b12 to neutralize a panel of 12 primary infant isolates was measured. Virus was obtained directly from infants at birth or within 2 weeks of age and was passaged once in PBMCs to produce viral stocks. Virus from these stocks was grown in PBMCs and neutralization assessed in a p24-based assay with PBMCs (*19*). IgG1 b12 achieved 90% neutralization for 8 of 12 isolates at concentrations ≤20 μg/ml (Table 2). All 12 isolates were 50% neutralized in the range 0.3 to 20 μg/ml, and most were neutralized at <5 μg/ml. In contrast, a pooled hyperimmune globulin product HIVIG (*20*) achieved 90% neutralization of

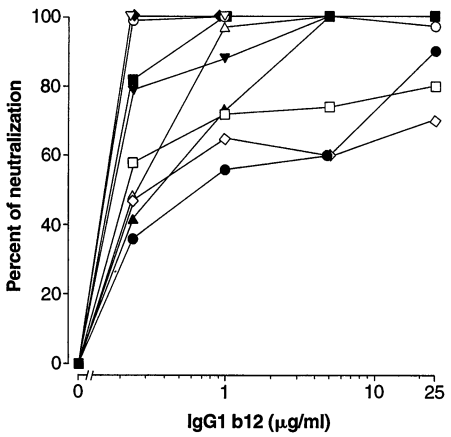

Fig. 1. Neutralization of primary isolates of HIV-1 by IgG1 b12. Virus neutralization was assessed with phytohemagglutinin (PHA)–stimulated PBMCs as indicator cells and determination of extracellular p24 as the reporter assay essentially as described (*17*). Virus [50 median tissue culture infectious dose ($TCID_{50}$)] and antibody at varying concentrations were incubated together for 30 min at 37°C before addition to PHA-stimulated PBMCs. Virus replication was assessed after 5 to 7 days by p24 ELISA measurement. The designation, location, and disease status of the virus donors were as follows: ■, VS (New York, acute); ▼, N_{70-2} (New Orleans, asymptomatic); ▲, AC [San Diego, acquired immunodeficiency syndrome (AIDS)]; ●, LS (Los Angeles, AIDS); □, NYC-A (New York, unknown); ▽, WM (Los Angeles, AIDS); △, RA (New York, acute); and ◇, JP (New York, acute). The molecularly cloned HIV-1 virus JR-CSF (◆) and HIV-1 isolate JR-FL (○) have been described elsewhere (*24*). Titers (50% neutralization) for the laboratory strains HIV-1 IIIB and HIV-1 MN (*25*) were <40 ng/ml.

Table 1. Neutralization of laboratory-adapted strains and primary isolates of HIV-1 by IgG1 b12 and a pooled human plasma preparation. The microplaque assay was carried out as described (*15*), with minor modifications. In brief, antibody was threefold serially diluted and preincubated in quadruplicate with an equal volume containing 20 plaque-forming units of virus per well for 18 hours in 96-well microtiter plates at 37°C. Thereafter, 90,000 MT-2 cells were added to each well and incubated an additional hour at 37°C. Assay medium containing agarose was added to each well to a final concentration of 0.8% agarose and the plates centrifuged to form cell monolayers. Plates were incubated for 6 days at 37°C and then stained with propidium iodide. After 24 to 48 hours, fluorescent plaques were counted on a transilluminator (304 nm). The neutralizing titer was defined as the concentration of antibody required to give a 50% or 90% reduction in plaque numbers as compared with controls containing no antibody. This dilution was interpolated between data points. VL134, VL648, and VL025 are viruses isolated from infected mothers in New York in 1992; UG274 and UG266 are clade D isolates (gift from J. Mascola, Division of Retrovirology, Walter Reed Army Institute of Research); the remaining viruses were isolated from homosexual males in California in 1992. The pooled human plasma preparation was derived from 13 HIV-1–positive individuals selected for high neutralization titer against the MN strain.

Virus	Host cell	IgG1 b12: neutralization titer (μg/ml)		Pooled human plasma: dilution for neutralization	
		50%	90%	50%	90%
IIIB	H9	0.007	0.04	1:767	1:270
MN	H9	0.003	0.91	1:24,000	1:8,000
VL135	PBMC	10	50	1:44	1:10
UG274	PBMC	0.7	11	1:37	<1:10
VL134	PBMC	5.6	17	1:30	1:10
VL596	PBMC	8.5	50	1:17	<1:10
UG266	PBMC	3.8	>50	1:12	<1:10
VL434	PBMC	22	200	1:10	<1:10
VL172	PBMC	>200	>200	1:10	1:10
VL750	PBMC	>200	>200	1:10	1:10
VL069	PBMC	>50	>50	<1:10	<1:10
VL077	PBMC	>200	>200	<1:10	<1:10
VL114	PBMC	<7.4	22	<1:10	<1:10
VL263	PBMC	5.0	17	<1:10	<1:10
VL648	PBMC	17	50	<1:10	<1:10
VL025	PBMC	17	>50	<1:10	<1:10

only 3 of 12 isolates within a concentration range up to 100 μg/ml.

To probe the occurrence of the b12 epitope in the HIV-1 pandemic, we examined binding of IgG1 b12 to gp120 from 69 international isolates belonging to six clades (*21*). IgG1 b12 reacted with ≥50% of clades A through D but only 1 of 12 isolates from clade E (Fig. 2). Reactivity with clade B isolates from the United States was approximately 75%.

Neutralization of HIV-1 by antibody shows considerable variation depending on the assay used and precise experimental conditions, such as inoculum size and incubation time of virus and antibody (*14*). However, by carrying out neutralization on a range of laboratory and primary isolates in a number of assays in different laboratories, we have shown that IgG1 b12 is a highly potent neutralizing antibody effective against a broad range of isolates. The results clearly demonstrate that, although primary isolates may be more difficult to neutralize by antibody than laboratory strains, they are not intrinsically resistant (*22*). The potency of IgG1 b12 against most U.S. isolates is within a concentration range (≤25 μg/ml) that could be achieved in vivo. Furthermore, the affinities of recombinant antibodies displayed on phage can be enhanced by mutagenesis and selection in vitro, and this strategy has been used to considerably improve the potency and range of reactivity of Fab b12 (*12*). For optimal potency and strain cross-reactivity for passive immunization, a cocktail of in vitro–improved antibodies may be most appropriate.

The results of our study have implications for vaccine design. The ability of IgG1 b12 to neutralize a broad range of primary isolates suggests that there may be conservation of a structural feature associated with the CD4 binding site of gp120 that is accessible to antibody and important for neutralization. A vaccine might seek to present this feature to the immune system. Clearly, the feature is present on recombinant gp120, because b12 was affinity selected from a library by means of this molecule. However, b12 and related antibodies formed only a small part of the repertoire of antibodies that were affinity selected from this library by recombinant gp120. Most of the antibodies obtained were far less potent in neutralization even though they were also directed to the CD4 binding site, competed with b12 for binding to recombinant gp120, and had affinities similar to b12 (*9–11*). Therefore, recombinant gp120 appears to present the b12 epitope in conjunction with several other weakly neutralizing and overlapping epitopes, and its efficacy as a vaccine may suffer as a result. Evidence from antibody binding to infected cells suggests that b12 may recognize a native conformation of gp120 more effectively than other antibodies directed against the CD4 binding site (*11*). In any case, IgG1 b12 and the approach using combinatorial phage display libraries could be useful in vaccine evaluation. The ability of a candidate vaccine to preferentially bind b12 or preferentially select potent neutralizing antibodies from libraries should be positive indicators for vaccine development.

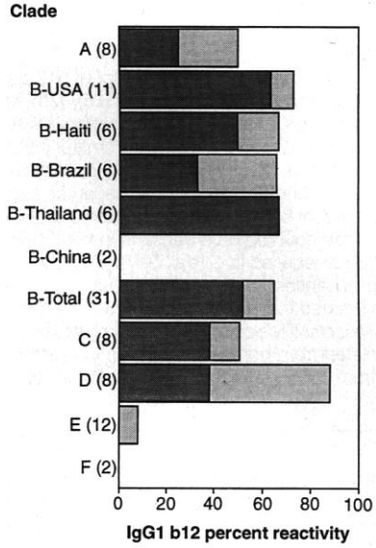

Fig. 2. Reactivity of IgG1 b12 with a panel of international isolates of HIV-1. Viruses were collected from various regions of the world (*21*), expanded in mitogen-stimulated PBMCs, (*4*) and culture supernatants containing infectious virus were stored in central repositories at −70°C. The designation of viruses into clades was made with sequence information based on the *gag* gene or on the V2-C5 region of gp120 or, in some cases, after heteroduplex mobility analysis (*26*). gp120 from culture supernates was captured with the use of a murine antibody to gp120 and IgG1 b12 reactivity was examined by ELISA (*27*). Dark shading, strong reactivity; light shading, moderate reactivity (*27*); and numbers in parentheses refer to the number of viruses from each clade that was examined.

Table 2. Neutralization of primary infant isolates by IgG1 b12. Neutralization was assessed with PHA-stimulated PBMCs as indicator cells and determination of extracellular p24 as the reporter assay essentially as described (*19*). Serial dilutions of IgG1 b12 (0.3 to 20 μg/ml) were incubated with 20 $TCID_{50}$ or 100 $TCID_{50}$ virus in triplicate for 2 hours at 37°C before addition to PHA-stimulated PBMCs. Virus replication was assessed after 5 days by p24 ELISA measurement. Neutralization was expressed as either a 50% or 90% reduction in p24 antigen as compared to values observed in the absence of antibody. Virus isolates were obtained from 12 infants born to HIV-1–seropositive mothers; 7 were obtained at birth and 5 between birth and 14 days of age. All the infants were from California. Virus was isolated from patient PBMCs by coculture with PBMCs from healthy seronegative donors. Viral stocks were prepared by passaging the last positive culture dilution once into PBMCs. All of the isolates, except one (isolate 7), were nonsyncytial inducing in MT2 cells.

Infant isolate	IgG1 b12: neutralization titer (μg/ml)	
	50%	≥90%
1	20	>20
2	1.25	>20
3	<0.3	0.3
4	<0.3	0.6
5	2.5	20
6	5	>20
7	5	>20
8	<0.3	0.3
9	0.3	5
10	0.3	2.5
11	<0.3	0.6
12	<0.3	0.3

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23. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; E, Glu; I, Ile; K, Lys; L, Leu; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; and V, Val.
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25. HIV-1 IIIB and HIV-1 MN are viruses with an extensive history of passage in transformed T cell lines [M. Robert-Guroff *et al.*, *Nature* **316**, 72 (1985)]. Stocks of these strains grown in H9 cells were passaged in mitogen-stimulated PBMCs to prepare viruses that had been grown in the same cells as the primary viruses, to eliminate the influence of any host cell–dependent epigenetic factors on virus neutralization (*5*). The stock of PBMC-grown MN was a gift from A. N. Conley (Merck Research Labs).
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27. Infectious culture supernatants containing virus and free gp120 were treated with 1% Nonidet-P40 nonionic detergent to provide a source of gp120 [J. P. Moore *et al.*, *AIDS* **3**, 155 (1989)]. An appropriate volume of inactivated supernatant was diluted with a buffer containing tris-buffered saline (TBS), 1% NP-40, 10% fetal bovine serum, and a 100-μl sample was added for 2 hours at room temperature to microplate wells (Immulon II, Dynatech) coated with sheep polyclonal antibody D7324 (Aalto Bio Reagents, Dublin, Ireland). This antibody was raised to peptide APTKAKRRVVQREKR (*23*), derived from the COOH-terminal 15 amino acids of the clade B IIIB isolate. Unbound gp120 was removed by washing with TBS, and bound gp120 was detected with CD4-IgG (1 μg/ml), or with monoclonal antibody, diluted in TMTSS buffer essentially as described [J. P. Moore *et al.*, *AIDS* **4**, 307 (1990); J. P. Moore *et al.*, *J. Virol.* **68**, 469 (1994)]. Bound ligand was then detected with an appropriate alkaline phosphatase–conjugated antibody to IgG, followed by AMPAK (Dako Diagnostics). Absorbance was read at 492 nm (A_{492}). Each virus was tested against CD4-IgG in triplicate and against IgG1 b12 in duplicate. All A_{492} values were corrected for nonspecific antibody binding in the absence of added gp120 (buffer blank). The mean, blank-corrected A_{492} values for CD4-IgG and IgG1 b12 were then calculated and the A_{492} ratios of IgG1 b12:CD4-IgG determined. This normalization procedure permits allowance to be made for the different amounts of gp120 captured onto the solid phase by D7324 when comparing antibody reactivity with a panel of viruses. Binding ratios of 0.50 or greater were deemed to represent strong antibody reactivity; ratios from 0.25 to 0.50 were considered indicative of moderate reactivity; values <0.25 were designated as representative of essentially negative monoclonal antibody reactivity.
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Crystal Structure of β-D-Cellotetraose Hemihydrate with Implications for the Structure of Cellulose II

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The crystal structure of β-D-cellotetraose shows the same molecular packing as cellulose II, with two antiparallel molecules in the unit cell. For cellulose II, the orientation of the C6–O6 bonds has been described as gauche-trans and trans-gauche, respectively, for the two antiparallel molecules, which otherwise have identical conformations. In contrast, in β-D-cellotetraose all C6–O6 bonds are gauche-trans, but the conformations of the two antiparallel molecules are different. Energy minimization and molecular dynamics studies suggest that the structure of cellulose II should be reinvestigated in light of these findings.

Although cellulose is the most abundant biological macromolecule and has been studied for 73 years with x-ray diffraction methods (*1*), there are still open questions concerning details of its three-dimensional structure. This holds for naturally occurring cellulose I and for cellulose II, which is obtained from cellulose I by treatment with alkali (mercerization).

In view of the fact that x-ray fiber diffraction techniques yield insufficient data for the complete description of a molecular structure at the atomic level, these techniques are usually complemented by model building. Using these methods, investigators have shown for cellulose I and II that the chainlike molecules are formed by (1→4)-linked β-D-glucopyranoses in the $^4C_1$ chair conformation, which are alternately rotated 180° along the chain axis. In cellulose I, all molecules are arranged parallel (*2*). In cellulose II, they are antiparallel and orientated along the unique *c* axis in the monoclinic unit cell with $a = 8.01$ Å, $b = 9.04$ Å, $c = 10.36$ Å, and $\gamma = 117.1°$, space group $P2_1$, with two molecules A and B in the asymmetric unit (*3–5*). Molecule A is located in the corner of the *a*, *b* plane of the unit cell and the antiparallel molecule B is in the center (Fig. 1) and shifted 2.24 Å (*3*) or 3.0 Å (*4*) along *c*. The torsion angles φ and Ψ, which describe rotation about the interglucose link, are identical in molecules A and B, but the orientation of the exocyclic C6–O6 bond, χ, has been described as *tg* (trans-gauche) for A and *gt* (gauche- trans) for B (*3*) [see (*6*)]. The conformation of cellulose II is stabilized by intramolecular hydrogen bonds O3···O5′ (A and B) and O2···O6′ (only A) (Table 1), and a number of intermolecular hydrogen bonds connect the molecules into sheets parallel to the (010) and (120) planes (Fig. 1). Because these interactions are not identical as reported in (*3*) and (*4*), the structure of cellulose II is still under debate and other models have been discussed, with major emphasis on the orientation of the C6–O6 bonds (*7*). One of the best methods to resolve the structural ambiguities would be x-ray diffraction on single crystals of oligo-β-D-celluloses. We describe here the crystal structure of β-D-cellotetraose (*8*), which exhibits a molecular

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