

The Refinement of the Haemagglutinin Membrane Glycoprotein of Influenza Virus

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Abstract

The crystal structure of the haemagglutinin glycoprotein, a trimer from the membrane of influenza virus, has been refined at 3.0 Å resolution to an *R* factor of 20.4%. The first 23 cycles were carried out on coordinates of an averaged monomer determined from a non-crystallographically threefold-symmetry-averaged electron density map. The contribution of structure factors to the least-squares equations were determined from non-crystallographically averaged gradient difference and curvature Fourier maps. The refinement was restrained using the Hendrickson & Konnert algorithm. These initial cycles were followed by 25 cycles of refinement with trigonometric evaluation of the derivatives on the complete trimer (208 422 daltons) on a Cray 1/S. Forty-eight water molecules and portions of five N-linked oligosaccharides were identified.

Introduction

The haemagglutinin of influenza virus (HA) is a virus-encoded integral membrane glycoprotein found at the surface of influenza virus. It is synthesized as a single polypeptide chain which is post-translationally cleaved into two chains (HA1 and HA2), covalently linked by one disulfide bridge. HA is the main antigen of influenza virus and has two known functions: it binds to a sialic-acid-containing receptor of infected cells and mediates the membrane fusion event required to transfer the viral nucleocapsid into the cytoplasm (for a review, see Wiley, Wilson & Skehel,

1984). The crystal structure of bromelain-released HA (BHA) from the A/Aichi/2/68 strain was studied at 3 Å resolution and the folding of the molecule described on the basis of an atomic model obtained by model building on an interactive graphics system (Wilson, Skehel & Wiley, 1981). In this paper, we describe the crystallographic refinement of a one amino-acid mutant (HA1 146 Gly → Asp) of that structure (Knossow, Daniels, Douglas, Skehel & Wiley 1984) (space group $P4_1$, $a = b = 162.6$, $c = 177.4$ Å).

To refine the atomic coordinates of BHA, we used a constrained refinement algorithm, based on Hendrickson's program (Hendrickson, 1981). Since the structure is a large one (208 422 daltons in the asymmetric unit), we needed to reduce the computing time for the initial steps of the refinement and this was done in two ways. The contribution of structure factors to least-squares equations was evaluated from difference and curvature maps, which were calculated using the FFT algorithm (Ten Eyck, 1977). We also took advantage of the non-crystallographic molecular threefold symmetry of the BHA trimer in the crystallographic asymmetric unit, and shifts were evaluated for a single monomer on averaged gradient and curvature maps (Bricogne, 1976).

In the first part, we will describe the computational method we used to evaluate the contribution of structure factors to the least-squares equations and the relationship of that method to others currently in use. We will then detail the course of the refinement and describe the results in terms of interpretability of the

maps, quality of the stereochemistry of the model and accuracy of the coordinates.

Computational method

The general method we used in the refinement is that developed by Hendrickson (Hendrickson & Konnert, 1980), which minimizes with respect to coordinates and temperature factors a composite observational function.

$$\begin{aligned} \varphi &= \sum_{\mathbf{h}} \sigma_F^{-2} (|F_{\mathbf{h}} \text{ obs}| - |F_{\mathbf{h}} \text{ calc}|)^2 \\ &\quad + \sum_I \sigma_I^{-2} (d_I \text{ ideal} - d_I \text{ model})^2 \\ &= \varphi_c + \varphi_s. \end{aligned} \quad (1)$$

\mathbf{h} is a vector in reciprocal space, d is a stereochemical restraint observation (bond distance, bond angle *etc.*) and σ is the variance of an observation.

The most time-consuming part of this process is the calculation of derivatives of φ_c with respect to atomic coordinates. The method we used to speed up this process is based on the observation (Cochran, 1951) that shifts can be calculated from a difference map. It has been shown (Agarwal, 1978) that shifts (Δx_m) of atomic coordinates in one refinement cycle can be calculated to a sufficient approximation by the formula

$$A\Delta\mathbf{x} = \mathbf{v},$$

where, if one considers only the structure-factor contribution to the least-squares equations and the diagonal elements of A (Hendrickson & Konnert, 1980),

$$\begin{aligned} Vx_m &= \sum_{\mathbf{h}} (|F_{\mathbf{h}} \text{ calc}| - |F_{\mathbf{h}} \text{ obs}|) (-2\pi i \mathbf{h}) g_m(s) \\ &\quad \times \exp i\varphi_{\mathbf{h}} \exp -2\pi i \mathbf{h} \cdot \mathbf{r}_m \end{aligned} \quad (2)$$

$$Ax_mx_n = \delta_{mn} \sum_{\mathbf{h}} 2\pi^2 h^2 g_m^2(s).$$

\mathbf{r}_m is the atomic position (x_m, y_m, z_m), \mathbf{h} the reciprocal-space vector (h, k, l) of modulus s , $g_m(s)$ is the atomic form factor including the temperature factor for atom m and $F_{\mathbf{h}} \text{ calc} = |F_{\mathbf{h}} \text{ calc}| \exp i\varphi_{\mathbf{h}}$, $\delta_{mn} = 0$ for $m \neq n$, $\delta_{mn} = 1$ for $m = n$. Similar formulae were given for $Ax_my_m, Ay_my_m, Vy_my_m$ *etc.*

The evaluation of V can be performed by computing the gradient of the difference map by numerical differences and then convoluting it with the atomic form factor at each atomic position (Jack & Levitt, 1978). A difference Fourier map is computed by FFT methods (Ten Eyck, 1977), but a noticeable amount of time is spent in calculating the numerical differences and then the convolution. In order to speed up calculations, we approximated the results of (2), neglecting one convolution by the form factor g , and used the following formulae to calculate the atomic

coordinate shifts:

$$\begin{aligned} A'\Delta\mathbf{x} &= \mathbf{v}' \\ A'x_mx_n &= \delta_{mn} \sum_{\mathbf{h}} g_m(s) 2\pi^2 h^2 \\ V'x_m &= \sum_{\mathbf{h}} (|F_{\mathbf{h}} \text{ calc}| - |F_{\mathbf{h}} \text{ obs}|) (-2\pi i \mathbf{h}) \\ &\quad \times \exp i\varphi_{\mathbf{h}} \exp -2\pi i \mathbf{h} \cdot \mathbf{r}_m \end{aligned} \quad (3)$$

Cochran (1948) has demonstrated that the approximation we use is equivalent to a least-squares solution in which each observation is weighted by the inverse of the appropriate atomic scattering factor. For the case of proteins refined at medium resolution (3 Å) and containing mostly C, N and O atoms, the atomic scattering factors are essentially identical and constant. Consequently, neglect of the convolution by g is equivalent to a least-squares solution in which each observation is weighted equally.

$V'x_m$ is the derivative of the difference map with respect to x at the position of atom m . A' can be evaluated from a 'curvature map', since the second derivative with respect to x of a calculated Fourier map at the position of atom m is

$$Cx_mx_m = -\sum_{\mathbf{h}} 4\pi^2 h^2 F_{\mathbf{h}} \text{ calc} \exp 2i\pi \mathbf{h} \cdot \mathbf{r}_m.$$

If the atomic contributions to $F \text{ calc}$ are detailed, this can be rewritten

$$Cx_mx_m = -\sum_j \sum_{\mathbf{h}} 4\pi^2 h^2 g_j(s) \exp 2i\pi \mathbf{h} \cdot (\mathbf{r}_j - \mathbf{r}_m).$$

The inner sum is the Fourier transform of a real and positive function which has its largest peak at the origin: making the same approximations as Agarwal (1978), we neglect it at all other points and the expression Cx_mx_m becomes

$$Cx_mx_m = -\sum_{\mathbf{h}} 4\pi^2 h^2 g_m(s).$$

This defines Fourier coefficients of a curvature map from which $A'x_mx_m$ is deduced. When one makes the hypothesis that atoms are spherical, values for all other combinations of the coordinates can be derived from the same map (Booth, 1946). Temperature-factor shifts were calculated with the same approximations as for coordinates, using the formulae:

$$\begin{aligned} V'_{B_m} &= \sum_{\mathbf{h}} (|F_{\mathbf{h}} \text{ obs}| - |F_{\mathbf{h}} \text{ calc}|) \exp i\varphi_{\mathbf{h}} (-s^2/4) \\ &\quad \times \exp 2i\pi \mathbf{h} \cdot \mathbf{r}_m \end{aligned} \quad (4)$$

$$A'_{B_m} = \sum_{\mathbf{h}} \frac{1}{2} g_m(s) s^4 / 16.$$

Gradient-curvature refinement has been used previously in protein crystallography (Moews & Kretsinger, 1975; Freer, Alden, Carter & Kraut, 1975), but the procedures employed made *a priori* estimates of the curvature based on the chemical nature of the atom examined. Here, shifts are deduced from

difference and curvature maps; this differs from methods shifting the atoms along the local steepest-descent direction of function φ_c (1) (Jack & Levitt, 1978) in that maps are not convoluted by g , the atomic form factor, to evaluate the shifts. The practical application to BHA which will be described illustrates a case where convergence was not noticeably slowed down by this approximation which allows a gain in computing time owing to the absence of convolution of maps by the atomic form factor.

Refinement

The starting model for refinement was obtained as described by Wilson, Skehel & Wiley (1981), the initial R factor being 37.5% for all data in the range 7.0 to 3.0 Å. In the first part of the refinement, shifts of the atomic coordinates were calculated for one BHA monomer using the gradient-curvature method and difference and curvature maps averaged around the non-crystallographic threefold trimer axis (Bricogne, 1976). In practice, curvature maps needed to be calculated once only or twice in each series of cycles, since curvatures were not found to vary significantly from one cycle to the next.

Stereochemical constraints were applied as described by Hendrickson & Konnert (1980) and relative weights of the various types of stereochemical constraints were as indicated by Hendrickson (1981). Contributions of structure factors were uniformly weighted so that the ratio of contributions of structure factors and the stereochemistry on the right-hand side of (1) were in the range 1.5 to 1.0. The higher values were used in the first cycles of each set of refinement cycles in order to give more weight to the structure-factor part of (1) and the ratio was gradually brought down to 1.0, in order to reach an acceptable stereochemistry of the model. Reflections contributing to refinement were in the resolution range 7 to 3 Å; in the 3.2–3.0 Å range, reflections were included only if they were larger than two standard deviations; reflections with $2(|F_{\text{obs}}| - |F_{\text{calc}}|) / (|F_{\text{obs}}| + |F_{\text{calc}}|) > 1.2$ were not included in the calculation of difference maps and therefore did not contribute to refinement, but they are included in R -factor calculations. After a series of 16 cycles, during which an overall temperature factor was used, the R factor was 27.3%, 4091 atoms being refined. After manual adjustment of the model using the program *FRODO* on an interactive graphics system (Jones, 1978), all atoms were refined with their temperature factor in a series of seven cycles. Temperature-factor shifts were calculated on a modified difference Fourier map (4) and refinement was constrained as described in (1). The R factor was 26.6% (see Table 1 for refinement parameters and results). Parts of the model which had a high temperature factor or where the stereochemistry was significantly

Table 1. *Refinement parameters and computer information*

Cycle	16	B7	T15	T25
R factor (%)	27.3	26.6	21	20.4
Number of parameters	12 254	16 337	49 345	49 541
atoms	4 084	4 084	12 336	12 336
temperature factors	1	4 084	12 336	12 385
water molecules	0	0	0	48
Number of observations*	80 668	91 044	138 411	138 671
R.m.s. shift between cycles quoted (Å)	0.77	0.55	0.71	0.37
CPU time per cycle	3 h 15 min†	13 min‡	55 min§	55 min§

* Including stereochemical constraints.

† Using a Vax 780.

‡ Using a Nas 7080.

§ Using a Cray 1/S.

distorted or which were involved in a crystal contact were then examined on a graphics system, using a non-averaged map, and manually refitted when possible.

In the second part of the refinement, the strict trimer threefold symmetry was released and a stereochemical non-crystallographic restraint was applied instead, all atoms of the trimer being refined. Structure factors and their derivatives were calculated trigonometrically. The strength of the threefold symmetry restraint was loosened for those residues which had one of their equivalents involved in a crystal contact (111 residues out of 1509 in a trimer) and which had been manually fitted to the electron density of a non-averaged ($2F_o - F_c$) map. After 16 cycles, the R factor was 21% when calculated for reflections as described above. Water molecules were located at positions where there were positive features larger than four standard deviations in a non-averaged difference map as well as positive features in a ($2F_o - F_c$) map in stereochemically reasonable positions. For those molecules which were not involved in a crystal contact, it was checked that they corresponded also to a strong feature in averaged maps and molecules related to them by non-crystallographic symmetry were added. A last series of ten refinement cycles was then calculated and water molecules which could be located on averaged maps (*i.e.* not located at a crystalline intertrimeric contact) were removed if their temperature factor was above a value of 30 \AA^2 . The final R value is 20.4% for 61 261 reflections. The r.m.s. deviations of stereochemical observations from their ideal values are: bond length 0.032 Å (target value 0.02 Å); deviations of atoms related by non-crystallographic symmetry after superposition 0.08 Å (target value 0.03 Å); peptide bond torsion angle 8.1° (target 3°).

Results

Progress of the refinement and interpretability of the maps

Of the 503 amino acid residues of one BHA monomer, only 15 are not located at the end of the

refinement. These are the NH₂ and COOH terminal residues 1–8 and 325–328 of HA1 and 173–175 of HA2. For other zones of the sequence which have a high temperature factor (average temperature factor for the structure 17 Å²; 100 residues in a monomer having atoms with a *B* larger than 25 Å²), the main-chain and most side-chain atoms could be assigned to definite density after cycle B7 (Table 1), the last cycle of gradient-curvature refinement. Phases at that stage were of sufficient precision to allow the location of a one amino acid change between HA A/Aichi/68 and the present structure (Knossow *et al.*, 1984). Fig. 1 represents one of the best defined zones of the molecule at the end of the refinement. At this stage, a significant number (37 residues in a monomer) of the least defined residues have at least one of their equivalents in the trimer involved in a crystal contact and must therefore be examined on non-averaged maps which, because they have a higher noise level than the corresponding averaged maps (standard deviation of a non-averaged $F_o - F_c$ map after cycle T25: 0.342 e Å⁻³; averaged map: 0.223 e Å⁻³), will always yield somewhat less accurate coordinates.

Precision of the results

Estimates of the error on coordinates were sought in two ways. The method of Luzzati (1952) gives an estimate of 0.4 Å for the overall mean position error, from the variation of the *R* factor with the resolution. This is an average value for the whole structure. The precision of coordinates may also be estimated from the r.m.s. deviations of the positions of atoms related by the trimer symmetry after superimposing the monomers in the asymmetric unit; this requires non-crystallographic symmetry restraint to be abandoned in the refinement. Since the resolution of the data

limits the number of observations, the non-crystallographic symmetry restraint was relaxed for only the part of the trimer at crystal contact sites, and therefore the values for the error deduced below on atomic positions are underestimates. They may nevertheless allow an appreciation of the variation of the precision in coordinates with the temperature factor. Statistics were done for atoms in well defined parts of the molecule (*B* lower than 10 Å²) where the r.m.s. positional error for main-chain atoms is 0.11 Å and for side-chain atoms is 0.13 Å. For atoms with a larger temperature factor (*B* larger than 25 Å², average *B* for these atoms 30 Å²), the r.m.s. positional error for main-chain atoms is 0.16 Å and for side-chain atoms is 0.22 Å.

Carbohydrate

HA is glycosylated at six positions on HA1 (residues 8, 22, 38, 81, 165 and 285) and one on HA2 (residue 154). Density was fitted with carbohydrate models at five of these positions (38, 81, 165, 285 of HA1 and 154 of HA2). The longest sequence built in the density is at residue 165, where four sugars are placed, out of eight possible residues (Fig. 2). All interactions of that oligosaccharide with the polypeptide chain, apart from those involving its first *N*-acetylglucosamine (NAG) residue, are with an HA monomer different from the one to which the chain is linked; they include van der Waals contacts of the first mannose residue with Trp 222 and a hydrogen bond between the O atom of the *N*-acetyl group of the second NAG residue and the NH of Trp 222. Hydrogen bonds between the hydroxyl in position 3 of a sugar and the ring O atom of the next sugar residue constrain the conformation of the chain to one similar to that found in the Fc fragment of human

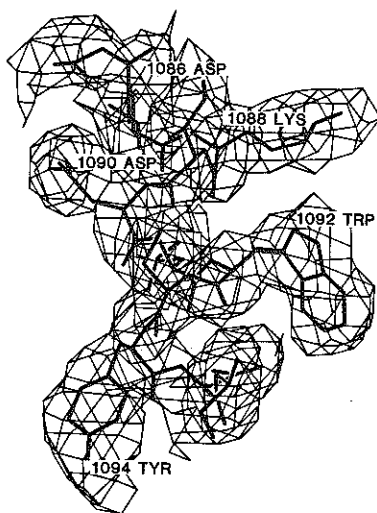


Fig. 1. The refined electron density map is shown superimposed on the model of nine residues (1086–1094) within an α -helix of the HA2 polypeptide chain, to demonstrate the map quality.

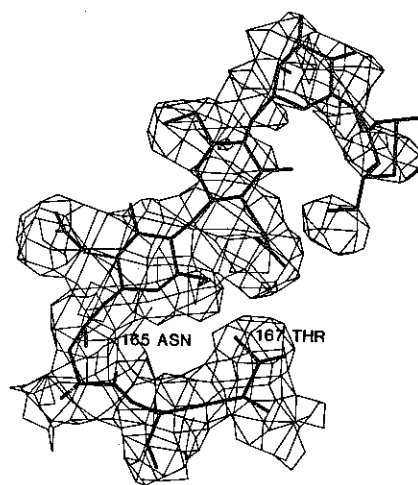


Fig. 2. Four residues of the *N*-linked oligosaccharide attached at Asn 165, two *N*-acetylglucosamines and two mannoses are shown superimposed on the refined electron density.

IgG (Deisenhofer, 1981; Wilson, Ladner, Skehel & Wiley, 1983). The contacts between the 165 carbohydrate and the polypeptide chain illustrate the possibility for carbohydrate to mask the polypeptide chain, altering the protein surface, and therefore affecting immune recognition, as has been observed (Skehel *et al.*, 1984).

Water molecules

Three water molecules were located at crystal contacts (intertrimeric) and 15 others were located for each monomer (these respect the threefold symmetry) and were kept in the final set of coordinates. Several of these water molecules relay interactions between different monomers in a trimer; among them are W 1191, which makes hydrogen bonds with OE2 of Glu 81 of one subunit and NE1 of Arg 70 of another subunit, and W 1195 which makes hydrogen bonds with NE2 of Arg 105 of one subunit and NE2 of Arg 106 of another subunit. But most remarkable, because of its low temperature factor (13.3 \AA^2) and the high peak in the difference Fourier maps at its location before it was introduced in the atom set, is

a water molecule which participates in the interactions that help maintain the NH_2 terminal part of HA2 buried in the trimer at $\text{pH} = 7$ (Daniels *et al.*, 1985). Water molecule W 1182 relays a hydrogen bond between the carbonyl O atom of Ile 6 of HA2 and NE2 of His 17 of HA1; in the same region, His 17 is held in the structure by another hydrogen bond through W 1183 to the carbonyl O atom of Arg 321 of HA1 (Fig. 3).

Trimer symmetry and crystal contacts

Non-crystallographic symmetry restraint was maintained through the whole refinement process for all residues, except those involved in a crystal contact (37 residues in a monomer). Circumstances may nevertheless arise, as has been shown to be the case for one of the insulin structures (Blundell *et al.*, 1972), where interatomic distances between atoms in different monomers of the same trimer do not allow the local molecular symmetry to be respected. At the end of refinement, 80 non-hydrogen-bond contact distances between different monomers of a trimer are shorter than 3.2 \AA . Some of the short contacts are between symmetry-related atoms and are potential candidates for such deviations from local threefold symmetry. Since these intratrimer contacts have no

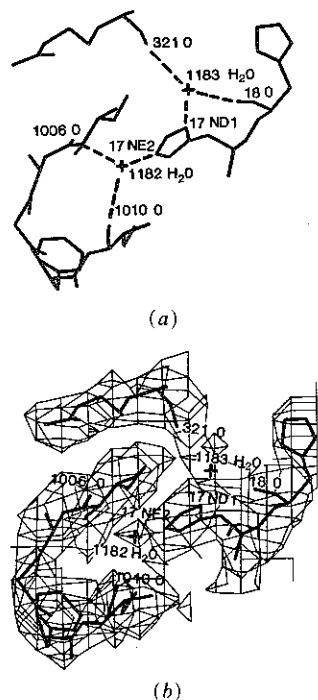


Fig. 3. Structural water molecules are found relaying hydrogen bonds in the vicinity of the amino terminal of HA2. (a) A schematic diagram showing water molecule 1182 relaying hydrogen bonds from His 17 (HA1) to the carbonyl O atoms of residues 6 and 10 (labelled 1006 and 1010) of the HA2 chain and water 1183 relaying hydrogen bonds from His 17 (HA1) to the carbonyl O atoms of residues 18 and 321 of (HA1). (b) The same view as in (a), showing the electron density for the protein and bridging water molecules.

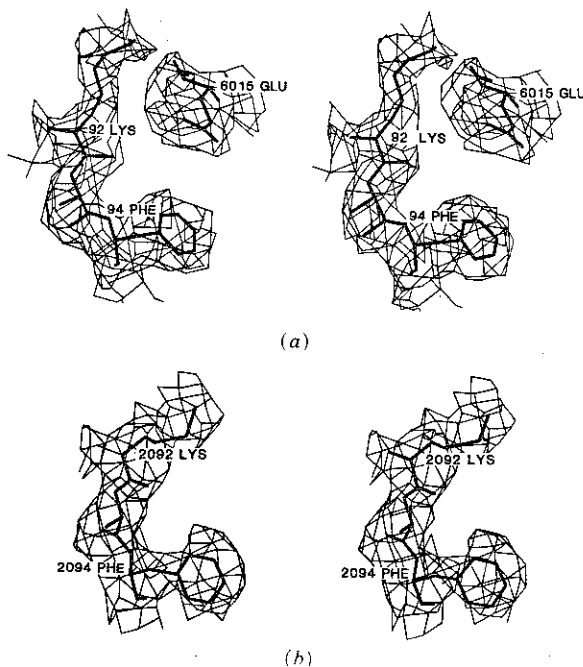


Fig. 4. Deviations from non-crystallographic symmetry are shown in the stereographic images of an intertrimeric crystal contact near residue 92 (Lys). (a) For the monomer involved in the contact, Lys 92 extends up away from a neighboring trimer residue 6015 (Glu). (b) The same region of chain as above, except on a non-crystallographically threefold-related monomer, which is not involved in a crystal contact. Lys 92 now extends to the right.

bearing on crystal interactions, the associated atoms would be statistically disordered. The resolution of the data and the small displacements involved to avoid these short contacts do not allow that disorder to be detected on maps or by inspection of temperature factors.

Crystal contacts are another cause for a deviation of the trimer from threefold symmetry. No major main-chain movement was found necessary to avoid unfavorable van der Waals interactions between trimers in a crystal (r.m.s. deviation of main-chain atoms for residues involved in a contact after superposition by the trimer symmetry: 0.24 \AA). However, Fig. 4 gives an example of a difference of conformation due to a crystal contact, of side chains of residues which are otherwise related by the trimer symmetry. Finally, another consequence of crystal contacts is a difference of the temperature factors of otherwise threefold equivalent atoms: e.g. residues 127–129 and 156–164 of HA1 have average temperature factors of 19 and 25 \AA^2 when they are at a crystal contact and of 25 and 32 \AA^2 otherwise (the r.m.s. deviation of temperature factors between atoms of these residues in the two equivalent monomers which are not involved in a contact is 0.6 \AA^2).

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The Structure and Absolute Configuration of (+)-Biperiden: a Chiral Ligand for the Pirenzepine Binding Site

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Abstract

The crystal structures of (+)-biperiden (*A*) and (+)-biperidenium L-(+)-mandelate (*B*) have been determined by X-ray crystallography. Crystal data: (*A*): $C_{21}H_{29}NO$, $M_r = 311.5$, orthorhombic, $P2_12_12_1$, $a = 5.8116(8)$, $b = 19.476(4)$, $c = 16.411(2) \text{ \AA}$, $V = 1857.5(5) \text{ \AA}^3$, $Z = 4$, $D_x = 1.11 \text{ g cm}^{-3}$, $\lambda(\text{Mo } K\alpha) = 0.071069 \text{ \AA}$, $\mu = 0.66 \text{ cm}^{-1}$, $F(000) = 680$, $T = 295 \text{ K}$, $R = 0.041$, $wR = 0.042$ for 1812 contributing reflec-

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tions; (*B*): $C_{21}H_{29}NO.C_8H_8O_3$, $M_r = 463.6$, monoclinic, $P2_1$, $a = 10.653(5)$, $b = 10.981(2)$, $c = 11.150(6) \text{ \AA}$, $\beta = 94.09(2)^\circ$, $V = 1301(1) \text{ \AA}^3$, $Z = 2$, $D_x = 1.18 \text{ g cm}^{-3}$, $\lambda(\text{Mo } K\alpha) = 0.071069 \text{ \AA}$, $\mu = 0.084 \text{ cm}^{-1}$, $F(000) = 500$, $T = 295 \text{ K}$, $R = 0.036$, $wR = 0.042$ for 1766 contributing reflections. The absolute configuration of (+)-biperiden is *S* as determined by correlation with the known configuration of L-(+)-mandelic acid. The (+)-biperiden ion conformation is superimposable on that of pirenzepine

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