Recognition of \( \beta \)-Ketoalcohol-derived Haptens by Tailor-made Antibodies

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Abstract: Antibodies steamed from mice immunized towards three different conjugates do not recognize the \( \beta \)-ketoalcohol for which they have been elaborated. They however recognize the haptens, which bear the side chain linking them to the carrier proteins. We have synthesized and tested for recognition a series of compounds related to those haptens, especially stereoisomers and compounds bearing part of their structures. Results and hypotheses are presented below.

Key words: antibodies, \( \beta \)-ketoalcohols, lipases, recognition, sulfur ylides

Few years ago we showed that the bicyclic dione 3 was a key intermediate in the stereoselective synthesis of racemic (Scheme 1) and of optically pure \( (1R) \)-chrysanthemic acids 1.

![Scheme 1](image)

It is readily available from dimethyl dimeredone 2 and possesses all the features required: (i) it has a cyclopropane ring bearing two substituents that are cis-one to each other allowing the stereoselective synthesis of the cis-chrysanthemic acid or its trans-stereoisomer by performing a further isomerization reaction (ii) it is prochiral and allows the synthesis of the (1R)- as well as the (1S)-series of chrysanthemic acid from which commercially valuable (1R)-cis- or (1R)-trans-chrysanthemic acids can be produced respectively.

The stereoselective reduction of 3 to the \( \beta \)-hydroxy ketone (1R)-4', which can either occur on (i) the exo- or endo-face of the concave bicyclic diketone 3 but also on (ii) either of the two carbonyl groups, is the key step of the whole process.1,2

Although each couple of endolexo-stereoisomers is formally able to generate one enantiomer of chrysanthemic acid, only those bearing an exo-hydroxyl group lead, in practice, to chrysanthemic acid after hydroxyl group activation (as a sulfonate) and further Grob fragmentation (Scheme 1).1,2

We have been able to generate2 the required optically pure (1R)-4' exo-stereoisomer (Scheme 2) and therefore the corresponding optically pure (1R)-chrysanthemic acids but the process involves few more steps. As an alternative, we thought of using tailor-made antibodies that catalyze the chemo- and enantioselective reduction of 3 to (1R)-4' by its most hindered face.

![Scheme 2](image)

A successful strategy requires generation of antibodies that would discriminate the four transition states leading to each of the stereoisomers.4,5 In order to get acquainted with this approach, we decided to generate antibodies able to recognize the (1R)-4' stereoisomer, precursor of (1R)-cis chrysanthemic acid, among a mixture of the four possible stereoisomers 4 (Scheme 2).

Antigens were designed in which the hapten, bearing the \( \beta \)-ketoalcohol possessing the exo-hydroxyl group and the bicyclic structure, is linked to a carrier protein by a saturated hydrocarbon linker (C-3 to C-8) attached: at the bicyclic structure side (i) directly to the endo-cyclopropyl methyl group or (ii) via a (O)-alkyloxime derived from the carbonyl group and at the protein side via (i) a disulfide linkage or (ii) a stronger amido group.
The (1R)-β-ketoalcohol 14 was first selected as the hapten because it possesses the bicyclic structure bearing the same functional groups and the same stereochemistry as the product (1R)-4 to be recognized. The presence of a thiol at the terminus of the side chain should allow to link it to the activated carrier protein OVA* produced from OVA by the SPDP method (Scheme 3).7

The synthesis of 14 involves (Scheme 4) the cyclopropagation of 5,5-dimethyl-cyclopent-2-en-1,3-dione 6 with the alkenylidenediphenylsulfuran 7 (DME, -78 °C, 1 h then 20 °C, 2 h, Scheme 4, step a). This reaction leads to a (80/20) stereoisomeric mixture of 8syn and 8anti in which the stereoisomer 8syn, bearing the alkyl chain in endo-position, prevails. Isolation of the major compound, whose stereochemistry was confirmed at a later stage by X-ray, 10 was readily achieved by preparative chromatography on SiO2.11
The synthesis of optically active (1R)-ketoalcohol 12 (Scheme 4, steps c-g) has been performed according to the procedure already disclosed for the synthesis of (1R)-4' from 3. It was achieved by (i) di-reduction of 8,9 from its more hindered endo-face using sodium borohydride in the presence of cerium trichloride (Scheme 4, step c), (ii) diacylation of the resulting diol 9 and (iii) enzymatic desymmetrization of the resulting diacetate 10 to 11 using Pig Liver Esterase (PLE, Scheme 4, step e). The transformation of 12 to 14 was achieved in two steps which involve (i) the addition of acetethylthiol via a radical process to terosorbent assay (ELISA), by antibody binding to solid-juvant. Two week after the last immunization, their sera were incubated with antigen coated on the plate (IC 50: > 500 µM). 18

We have then repeated the above mentioned series of experiments using instead for immunization the OVA*-17 antigen derived from the hapten 17 which possesses the same linker as the one present in (1R)-14 but attached at another site on the bicyclic structure (Scheme 3 and 5) and found similar results. The β-ketoalcohol (1R)-4' is unable to inhibit the fixation of antibodies AB-OVA*-17 on BSA*-17 (IC 50 > 500 µM) whereas the alkoximinocohol (1R)-19c (R = Pent, Scheme 5), which possesses the bicyclic structure as well as a side chain of critical length, is able to inhibit AB-OVA*-17 (IC 50: 50 µM).

We have also carried out related experiments involving antigens KLH-18 possessing a more stable linker than the disulfide group, used for OVA*-14 and OVA*-17, and using a more immunogenic carrier protein : the Keyhole Limpet Hemocyanin (KLH). 21

The conjugate KLH-18 was designed and prepared (Scheme 3) from KLH and the β-alkoximinocohol (1R)-18 produced stepwise from (1R)-4', hydroxylamine, 1,5-dibromopentane, potassium thiolacetate and PLAP (Scheme 5, steps a-e). BA-18 which was also required for ELISA was produced accordingly.

Antibodies AB-KLH-18 raised against KLH-18 and assayed on the conjugate BSA-18 17d possess a very poor affinity toward the β-ketoalcohol (1R)-4' either (IC 50 > 500 µM, Figure 2, •). 18 However, they were highly specific to the alkoximinocohol (1R)-19b (R = n-Bu, Scheme 5) which possesses the bicyclic structure as well as a side chain of critical length related to the linker (IC 50: 8 µM, Figure 2, □). 18

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The same antibodies binds more efficiently the β-ketoalcohol (1R)-12, which possesses part of the linker joining (1R)-4' to OVA (IC 50: 250 µM).

Four Balb/c mice were immunized with OVA*-14 (i) once with 100 µg in the presence of complete Freund adjuvant then (ii) three times consecutively with 50 µg of the same OVA*-14 in the presence of incomplete Freund adjuvant. Two week after the last immunization, their sera have been collected and tested by enzyme linked immunosorbent assay (ELISA) by antibody binding to solid-phase bound BSA*-14 antigen. BSA*-14 was in turn produced by coupling of (1R)-14 to activated bovine serum albumine (BSA*), instead of OVA* (Scheme 3).

Although the presence of immunoglobulins specific to the hapten 14 or part of it, in the sera of immunized mice was confirmed by indirect ELISA (Figure 1, •), we were somewhat rather surprised, using an inhibition ELISA, to find that the related β-ketoalcohol (1R)-4' did not inhibit the fixation of AB-OVA*-14 antibodies to the BSA*-14 antigen coated on the plate (IC 50: > 500 µM). 18

![Figure 1](image-url) Indirect ELISA: Titration Curves for Various Antibodies

![Figure 2](image-url) Inhibitive ELISA: Inhibition Curves for Various Inhibitors Implying AB-KLH-18
This behavior is similar to those reported above from AB-OVA*-14 and AB-OVA*-17 instead. The presence on the substrates of the carbon framework of the linker was essential, in all the three cases reported (AB-OVA*-14, AB-OVA*-17 and AB-KLH-18) for their recognition.

We therefore decided to test the affinity of the antibodies raised against KLH-18, to complex a series of substrates such as (1S)-4', (1R)-4', 15, 19b', 20, 21, 23, 27 and 30 (Schemes 5 and 6), whose structures are some-

what related to that of the ketoalcohol (1R)-4'. Some of their syntheses are gathered in Schemes 5 and 6.

We first found that antibodies AB-KLH-18 neither recognize the regioisomeric endo-ketoalcohol (1R)-4' nor the enantiomeric exo-ketoalcohol (1S)-4' (Scheme 2, IC₅₀ > 500 µM).

We also found that these antibodies did not bind efficiently the O-unsubstituted oxime 15, which possesses all the functional groups present on the hapten (IC₅₀ > 500 µM, Figure 2, ), but bind effectively its O-ethyl-substituted homologue 19a (IC₅₀ : 32 µM). Increasing the length of the side chain to O-Bu as in 19b (IC₅₀ : 8 µM, Figure 2, x) leads to an increasing inhibition. This clearly confirms, as we described above, that the linker plays an important role in the recognition process.

We have also observed that the presence of a carbonyl group on the side chain of sufficient length (as in 20a), has a little effect on the inhibition and this is also the case for compounds 20b-d,f bearing a carboxy group at the terminus of the side chain (IC₅₀ : 32 µM for X = C(=O)NH₂, C(=O)NMe₂ and C(=O)OH; 16 µM for X = C(=O)OMe).

Interestingly 20e, bearing the ester group at the terminus of the side chain is a better ligand (IC₅₀ : 16 µM) than 21 (IC₅₀ : 128 µM), in which the same functional group is internal.
As expected from the previous results, neither 19b, the diastereoisomer of 19b, bearing a hydroxy group in endo-position, nor 19b, the enantiomer of 19b are recognized by the antibodies (IC50 > 500 μM).

We have finally observed that the latter were highly specific to the whole structure present on the hapten KLH-18 since they did not recognize 23 missing the bicyclic structure present on 4, nor 27 missing the cyclopropane ring. Even more surprising, they were unable to bind 30endo and 30exo on which a single exo- or endo-methyl group is missing (IC50 > 500 μM).

We have shown that the sera steamed from mice immunized towards three different conjugates: OVA*-14, OVA*-17 and KLH-18, do not recognize (1R)-44 for which they have been elaborated. We do not understand the real reasons of such observation. Haptens 14, 17 and 18 present on the conjugates OVA*-14, OVA*-17 and KLH-18 are all small molecules. Although some, even closely related conjugates, have been successfully used to produce antibodies which recognize small molecules without apparent trouble related to the linkers, it remains that most of the published examples involve not only substrates but also haptens bearing aromatic rings and polar groups. This is not the case of our conjugates. We are pursuing our study towards these goals.

References and Notes

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(7) This method offers the advantage to measure, by fluorimetry, the number of hapten attached to the protein.7 (a) Carlsson, J.; Drevin, H.; Axen, R. Biochem. J. 1978, 173, 723.
(9) The diphenyl sulfuran 7 was prepared by metallation of the corresponding sulfonium tetrafluoroborate with LDA (1 equiv, CH2Cl2, DME, -78 °C, 0.5 h) The latter salt was prepared by methylation of 1-(4-pentenylidene)diphenyl sulfuran and was directly used in the next step.
(10) X-ray has been performed on the camphanato of 11 (Scheme 4, Mp 117 °C).
(11) The reaction of the same ylide 7 on 6,6-dimethyl-(R)-4-acetoxy-cyclopent-2-en-1-one is more convergent and provides directly after deacetylation to optically active 12 in high yield but as an intractable mixture of syn- and anti- stereoisomers.
(13) Destruction of the tetractate moiety cannot be properly achieved in basic media because bases also react on the aldol functional group present on the bicycle. Furthermore the thiolate produced, in such medium, is very air sensitive. It is therefore very difficult, since the reaction is carried out on small quantities, to avoid the concomitant formation of the corresponding disulfide.
(16) (a) General procedure for the indirect ELISA8a: Calculated concentration of antigen16e was coated (24 h, 4 °C) on the wells of a polystyrene microtiter plate. After blocking of unoccupied adsorption sites on the polymer surface, mouse serum was added in various dilutions to the wells and incubated (24 h, 4 °C). After washing of the wells, enzyme-labelled antibody specific for mice antibodies was added. After further incubation (1 h, 37 °C) and washing, the corresponding chromogenic enzyme substrate was dispensed. The coloured product that indicates the amount of the first antibody bound to the solid-phase immobilized antigen was determined by simple photometrical absorption at 490 nm and 630 nm. Between all steps, excess reagent are removed by washing with a PBS-Tween 20 (0.1% solution) (b) General procedure for the inhibition ELISA: Calculated concentration of antigen16e was bound to the wells of a polystyrene microtiter plate. After blocking of unoccupied adsorption sites on the polymer surface, optimal dilution of mouse serum (determined via indirect ELISA test described above)8a was incubated 2 h at 37 °C with the test samples (inhibitive antigen) and was added to the wells. Next steps are the same as described above. (c) Crower, J. R. Methods in Molecular Biology 1995, p 42.
(17) Measured optical densities for: (a) Non immunized mice (NIM, Figure 1, •) (b) AB-OVA*-14: Figure 1, ◆ (c) AB-OVA*-17: Figure 1, □ (d) AB-KLH-18: Figure 1, Δ.
(18) A single example is presented for convenience. Three others have been studied which exhibit similar patterns.
(19) Enantioomerically pure 17 has been prepared from 4 via the oxime 15. 15 has been transformed to 16 by sequential reaction with only one equivalent of potassium tert-butoxide and excess (1.5 equiv) of 1,5-dibromopentane.29 16 was then transformed to 17 on reaction with potassium thiolactate and hydrolysis of the resulting alkyliithiolydic acid with PLAP as disclosed above (Scheme 4, step i). The oxime 15 has been in turn prepared on reaction of the β-ketoalcohol 4 with hydroxylamine hydrochloride and sodium acetate in methanol at reflux. Its structure has been unambiguously assessed by X-ray crystallography of its di-N(O-4-bromobenzoate) which...
shows that the N-O bond lies away from the gem dimethyl group on the cyclopentane ring.


(22) The key step of 18, 19, 20 and 21 takes advantage of the selective O-alkylation of the oxime\(^{20}\) performed as described above.\(^{19}\) The synthesis of the activated ester 18 has been achieved by DCC coupling of the corresponding acid with \(N\)-hydroxysuccinimide in the presence of catalytic amounts of DMAP.

(23) The \textit{endo} hydroxyl derivative 19' has been synthesized from its \textit{exo}-stereoisomer on oxidation to a ketoxime which has been then chemo- and stereoselectively reduced from its \textit{exo}-face by lithium triethylborohydride.\(^{1,2}\)

(24) The synthesis of the cyclopentanol 27 as well as of the bicyclic derivatives 30 bearing a methyl group in \textit{endo}- or \textit{exo}-position has been achieved from optically pure 24\(^{4}\) by catalytic hydrogenation or cyclopropanation with ethyldiene diphenylsulfurane respectively. 28 is obtained as an untractable mixture (50/50) of stereoisomers. The related oximes 29 have been nevertheless easily separated and each stereoisomer has been transformed to the \(O\)-butyl oxime as described above.\(^{19,20}\)
