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Feasibility of an Immunoassay for Mevalonolactone

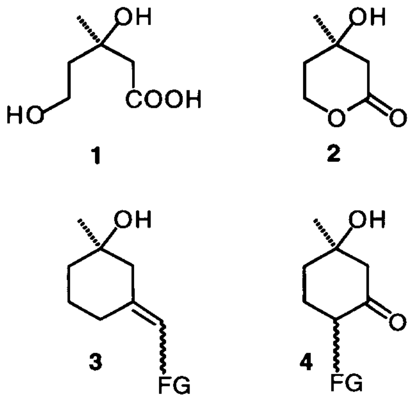
Thomas A. Spencer,[a,*] Dawn S. Clark,[a] Gary A. Johnson,[a] Sandra K. Erickson[b] and Linda K. Curtiss[c]

[a]*Department of Chemistry, Dartmouth College, Hanover, NH 03755, U.S.A.*
[b]*Department of Medicine, Veterans Administration Medical Center and University of California, San Francisco, CA 94121, U.S.A.*
[c]*Departments of Immunology and Vascular Biology, The Scripps Research Institute, La Jolla, CA 92037, U.S.A.*

Abstract—Mevalonic acid is a key intermediate in a broad spectrum of cellular biological processes and their regulation. Availability of a rapid, sensitive and accurate method for its assay would be highly useful. Therefore, the feasibility of developing an immunoassay for mevalonic acid in biological samples was explored. The strategy employed was to synthesize several racemic haptens structurally resembling *R*-mevalonolactone, the cyclic form of mevalonic acid present at lower pH and presumed to be more antigenic. Two of these haptens were coupled to keyhole limpet hemocyanin, and the resulting conjugates were used successfully to generate antibodies in rabbits. The first antiserum bound to *R*,*S*-mevalonolactone much more effectively at pH 4.0 than at pH 6.0, consistent with the structural resemblance of the haptens to the lactone form. This antiserum also bound the free hapten from which it was generated and two others of different structure with comparable effectiveness, and slightly better than it bound *R*,*S*-mevalonolactone at pH 4.0. Similar results were obtained with the antiserum to the second hapten. The binding of either antiserum to the natural enantiomer, *R*-mevalonolactone, was 20 times weaker than to *R*,*S*-mevalonolactone, suggesting that the nonbiological enantiomer was more antigenic. Nevertheless, the results demonstrate that an immunochemical approach to accurate quantitation of mevalonic acid in biological samples is feasible. © 1997 Elsevier Science Ltd.

Introduction

Mevalonic acid (MVA, **1**, Scheme 1) is a key intermediate in cellular biochemistry.[1-3] It is a precursor for a number of biologically important lipids, including cholesterol, steroid hormones, bile acids, ubiquinone and dolichols. In addition, mevalonate-derived isoprenoids are intermediates in the biosynthesis of isoprenylated tRNAs, prenylated proteins involved in cell signaling and growth, and heme **a**, a prosthetic group of cytochrome oxidase. Inhibition of cellular MVA production leads to induction of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase[4] and low density lipoprotein receptor,[5] cell cycle arrest,[6,7] or apoptosis.[8-10] In the intact animal, including man, it leads to lowering of plasma cholesterol.[11-13] HMG-CoA reductase inhibitors are used to treat hyperlipidemia, and they have been proposed as antineoplastic agents. A variety of studies in cell culture[14-18] and in tumor-bearing animals[19] suggest they may be useful in the chemotherapy of some types of cancer. Plasma MVA levels correlate with whole body rates of cholesterol synthesis in both experimental animals and man.[20] Urinary MVA levels correlate with plasma levels,[21] and have been used to study the mechanisms of hyperlipidemias,[22] the efficacy of treatment of hyperlipidemias,[23-25] and the efficacy of lovastatin as an anticancer agent in phase I trials.[26] Thus, development of methods for the accurate and convenient quantitation of MVA in biological samples is of considerable importance, both as a research tool and for clinical use in treatment of hyperlipidemias and, potentially, malignancies.



Scheme 1. Structures of: *R*-mevalonic acid (**1**); *R*-mevalonolactone (**2**); two generalized prospective hapten types **3** and **4** for **2**, with a spacer arm of unspecified length bearing an unspecified functional group (FG) for attachment to a protein.

A number of different approaches have been used to develop assays for MVA. Several methods involving gas chromatography–mass spectrometry (GC–MS) have been reported, beginning with that of Hagenfeldt and Hellström in 1972.[27] Improved GC–MS methods were developed by Del Puppo et al.[28] and by Scoppola et al.,[29] and recently by Lindenthal and von Bergmann[30] and by Ishihama et al.[31] However, all these require complex and expensive instrumentation. Very recently, a procedure for acid-catalyzed conversion of MVA to dehydromevalonolactone, HPLC separation and UV detection was described by Gondoh et al.[32] The most widely used method has been the enzymatic phosphorylation procedure of Popjak et al.[33] Although this method is specific and sensitive, it is lengthy, requiring a number of steps and the use of $[\gamma^{32}P]$ATP.

We sought a method of analysis for MVA that would be sensitive, specific, reliable and convenient to use. An immunoassay seemed to be an attractive choice to satisfy those aims. The fact that MVA (**1**) is in facile equilibrium with *R*-mevalonolactone (MVL, **2**), as shown in Scheme 1, presented certain unusual considerations with respect to the development of an immunoassay. The cyclic lactone form **2** is an attractive structure to mimic in a synthesized hapten because it is more distinct and conformationally more rigid than the open-chain structure **1**, thus rendering it more likely to be immunogenic.[34] On the other hand, at serum physiologic pH, essentially all the MVA in any sample would exist as the carboxylate anion of **1**, rather than as lactone **2**, so that an immunoassay based on antibodies to haptens resembling **2** might require that the assay be conducted at pH$<$5. Because such conditions should in theory not pose a problem with respect to antigen–antibody interactions, it was decided to use the lactone form **2** as the model for a suitable hapten. Preparation of the first haptens chosen, the 4-hemisuccinate esters of *cis*- and *trans*-1-methylcyclohexane-1,4-diol, has been described previously.[35] In this paper the synthesis of two additional types of haptens, cyclohexane derivatives **3** and **4** (Scheme 1), and their use in assessing the feasibility of creating an immunoassay for MVA are described.

Results and Discussion

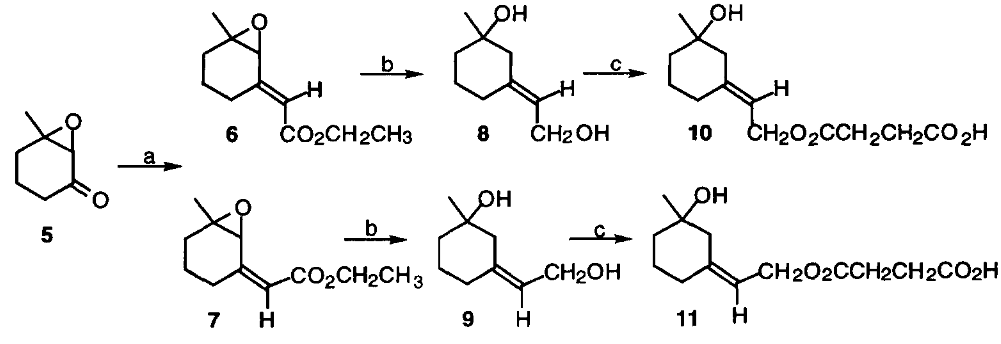
Hapten synthesis

For practical reasons, the syntheses were conducted in a nonasymmetric manner to afford racemic samples of haptens **3** and **4**. These were then conjugated with a suitable protein to develop antibodies. Screening for antisera selective for *R*-mevalonolactone (**2**) was accomplished by determining which antibodies recognized separately synthesized samples of **2**.[36]

Haptens of type **3** were synthesized from known[37] epoxyketone **5** by the series of reactions depicted in Scheme 2. A modified Horner–Emmons–Wadsworth reaction of **5** by a procedure of Baggiolini et al.[38] afforded 90% of an approximately 1:1 mixture of **6** and **7**. It was originally intended to separate the two series of geometric isomers at a later stage in the synthesis, but it eventually proved necessary to use laborious silica gel chromatography to effect separation of **6** and **7** in order to get pure samples of representatives of both the **Z** and **E** series. Assignment of structure to **6** vs. **7** was easily made on the basis of the deshielding by the ester carbonyl group of the epoxide proton of **6** (δ 4.53) relative to the epoxide proton of **7** (δ 3.12 ppm), exactly as had been observed in a pair of closely related geometric isomers.[39] Pure **6** and **7** were then individually reduced with lithium aluminium hydride to afford 65% of diol **8** and 63% of diol **9**, respectively. The primary hydroxyl group of each diol was then selectively esterified with succinic anhydride to afford haptens **10** and **11**, with the hemisuccinate spacer arm ready for attachment to a protein.

Synthesis of a hapten of type **4** proved more challenging. The initial and unsuccessful approach to introduction of the C6 spacer arm in **4** involved attempted alkylation with an ω-haloalkene of the dianion derived from the known[40,41] 3-hydroxy-3-methylcyclohexanone. Next, the trimethylsilyl enol ether[42] of epoxyketone **5** was employed as an intermediate in attempted application of Marino's interesting S_N2' cuprate substitution reaction,[42] and as a precursor of the enolate anion required for C6 alkylation,[43] but both of these strategies likewise failed.

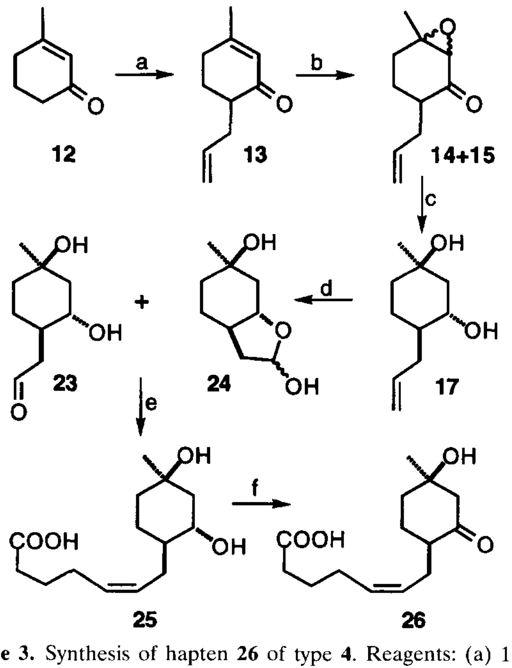
Success in preparing a type **4** hapten was finally achieved via alkylation of 3-methylcyclohex-2-en-1-one (**12**) (Scheme 3). Although the anion formed from **12** with lithium diisopropyl amide afforded only extremely poor yields of desired C6 alkylation with ω-haloalkenes, the known reaction[44] with allyl bromide[45] afforded 80% of **13**. Epoxidation of **13** with basic hydrogen peroxide then gave 86% of a 3:2 mixture of diastereomeric epoxyketones **14** and **15**. Reduction of this mixture with lithium aluminum hydride gave an approximately 1:2:1:1 mixture of all four possible diastereomeric diols **16**–**19** (listed in order of decreasing TLC R_f values);



Scheme 2. Synthesis of haptens **10** and **11** of type **3**. Reagents: (a) $EtO_2CCH_2PO(OEt)_2$, NaH, THF; (b) $LiAlH_4$, Et_2O; (c) succinic anhydride, Et_3N, THF.

from which about 85% of the major isomer **17** present crystallized upon standing (Scheme 4). Assignment of structures to the four isomeric 1-methyl-4-(2-propenyl)-1,3-cyclohexanediols **16–19** was accomplished as summarized in Scheme 4. First, each of pure **16** and **17** and the inseparable mixture of **18** and **19** was oxidized by the method of Griffith et al.[46] to the corresponding ketone, **20** or **21**, which was then reduced back to diol with LAH. These sets of conversions produced from each of **16** and **17** a 4:1 mixture of **16** and **17**, and from the mixture of **18** and **19** a 1:1 mixture of **18** and **19**, thus establishing that **16** and **17** (and therefore **18** and **19**) had the same stereochemistry at C1. The exceptional TLC mobility of **16** suggested that it was an intramolecularly hydrogen-bonded 1,3-diaxial diol, and this was confirmed by its conversion to cyclic carbonate derivative **22**. The ^{1}H NMR spectrum of diol isomer **17** showed the methane proton (band width 30 Hz) as a doublet of triplets at d 3.62 in $CDCl_3$ with one axial–equatorial coupling constant of about 4.1 Hz and two axial–axial coupling constants of about 10.2 Hz.

These data are consistent only with the structural assignments given in Scheme 4. If, for example, the carbonate derivative assigned as **22** were instead in fact derived from conformation b of isomer **19**, then the axial methine proton in **18b** would show two axial–equatorial couplings and one diaxial coupling. Further confirmation of the correctness of the structural assignments was obtained from the NMR behavior of the mixture of **18** and **19**. The methine protons signals for **18** and **19** in $CDCl_3$ at δ 4.00 and δ 3.50 both had a width of 21 Hz. When the solvent was switched to D_2O, the methine proton signal at δ 4.00 broadened slightly, but the other now appeared at δ 3.32 with a band width of 30 Hz as a doublet of triplets with J = 4.2, 10.4 and 10.4 Hz. These observations are consistent with a solvent-dependent equilibrium between conformations **19a** and **19b**, with the intramolecularly hydrogen-bonded 1,3-diaxial diol **19b** dominating in $CDCl_3$, and **19a**, having an axial methine proton, dominating in D_2O. Closely parallel observations were made by Nader et al.[47] in the case of the 1-methyl-cyclohexane-1,3-diols. The fact that two chair conformations of similar energies are observed for **19** is also consistent with the stereochemical assignments to **18** and **19** vs. **16** and **17**, since **18** or **19** will have one alkyl group equatorial and one axial in either conformer, whereas **16** and **17** have both alkyl groups equatorial in the presumably dominant conformers shown in Scheme 4.

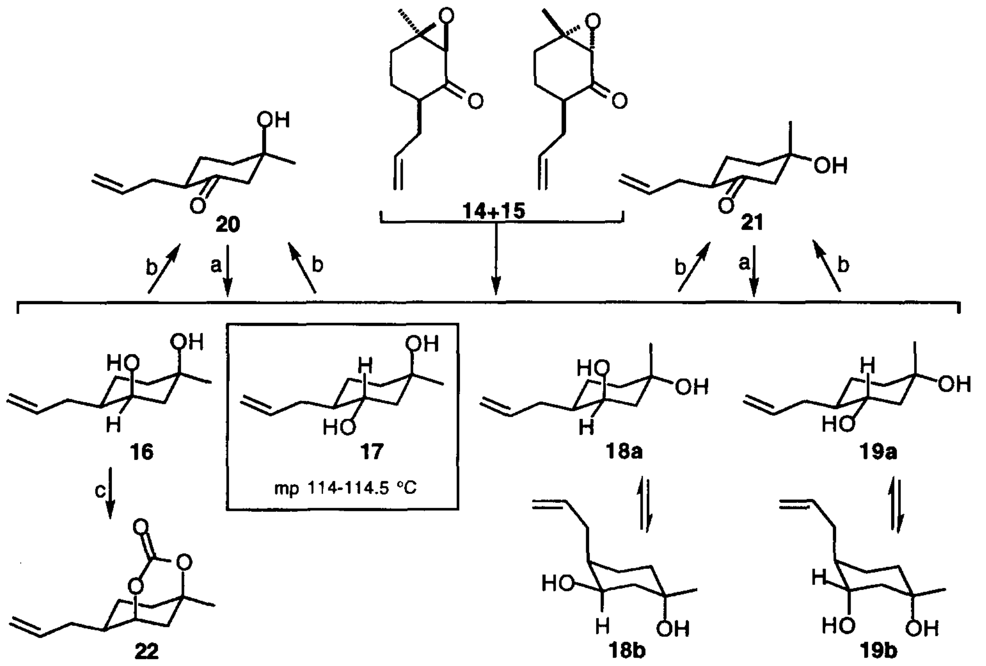


Scheme 3. Synthesis of hapten **26** of type **4**. Reagents: (a) 1. LDA, THF, 2. $H_2C{=}CHCH_2Br$; (b) H_2O_2, H_2O, CH_3OH, K_2CO_3; (c) $LiAlH_4$, Et_2O; (d) O_3, CH_2Cl_2, CH_3OH; (e) $[Ph_3P(CH_2)_4CO_2H]Br$, $KN(SiMe_3)_2$, THF; (f) NMNO, tetra-*n*-propylammonium perruthenate, CH_2Cl_2.

Completion of synthesis of a type **4** hapten was conducted with the crystalline diol isomer **17**, as depicted in Scheme 3. Ozonolysis of **17** afforded 87% of aldehyde **23**, which was obtained to the extent of 20% in the isomeric hemiacetal form **24**. This mixture of **23** and **24** was then subjected to a Wittig reaction with the ylide from (4-carboxybutyl)triphenylphosphonium bromide to afford **25**, which was oxidized[46] without purification to afford prospective hapten **26**. That **25** and **26** were, as expected,[48] the *Z* isomers was confirmed by analysis of the 500-MHz ^{1}H NMR spectrum of **26**, as described in the Experimental section, which showed that the alkenyl protons have J = 10.6 Hz.

Immunoassay for mevalonolactone

The immunogenicity of haptens **10** and **11** was determined by conjugation to keyhole limpet hemocyanin (KLH), followed by immunization of rabbits as described in the Experimental section to obtain the two specific polyclonal antisera used in these studies. Both antigens induced the production of high-titre antibodies, which reacted with the immunogen in solid-phase immunoassays at antisera dilutions of 10^{-4}–10^{-5}. Specificity of the antisera for free haptens was examined by competitive immunoassay. These assays were performed by measuring the capacity of the free haptens in solution to interfere with the binding of a fixed and limited amount of each antiserum to immobilized hapten–KLH conjugates. As discussed above, both hapten–KLH immunogens had been based on the cyclic MVL (**2**) rather than on the open-chain MVA (**1**); therefore the likelihood that the antisera would recognize MVA only at a pH at which the lactone form **2** would be present was examined by studying the effect of pH on the ability of *R*,*S*-MVL to compete in the immunoassay. No antibody binding of *R*,*S*-MVL was observed at pH 3.0, but binding (IC_{50} = 1.10 mg/mL) was obtained at pH 4.0. This binding at pH 4.0 was significantly greater than that detected at pH 5.0 (IC_{50} = 10.2 mg/mL) or at pH 6.0 or 7.0 (IC_{50} > 50 mg/mL), consistent with the idea that the lactone form was being recognized.



Scheme 4. Structural identification of the four isomeric diols produced by reduction of epoxyketone mixture **14** + **15**. Reagents used: (a) $LiAlH_4$, Et_2O; (b) NMNO, tetra-*n*-propylammonium perruthenate, CH_2Cl_2; (c) *N*,*N'*-carbonyldiimidazole, benzene.

The binding of racemic haptens **10**, **11** and **26** at pH 4.0 to the antiserum generated by immunization with hapten **11**-KLH was next evaluated, and the results are shown in Figure 1. Each of these three free haptens showed indistinguishable binding, with an IC_{50} value of 152 μg/mL. This result strongly suggests that this antiserum includes antibodies that recognize the common ring structure element present in these three haptens. Also shown in Figure 1 is the previously mentioned finding that racemic mevalonolactone (*R*,*S*-MVL) binds at pH 4.0 to this antiserum.

Next, experiments were conducted to determine whether the antisera would bind the natural enantiomer, *R*-MVL (**2**). The antiserum generated by immunization with the racemic **10**-KLH conjugate was compared with that from the racemic **11**-KLH conjugate in binding competition with the corresponding free hapten (**10** or **11**, respectively), *R*,*S*-MVL, and *R*-MVL (**2**). The results are shown in Figure 2. The IC_{50} values for these competitors with the antiserum to **10**-KLH were 160, 690 and 13,400 μg/mL, respectively. With the antiserum to 11-KLH, they were 140, 690 and 15,000 (an extrapolated value) μg/mL, respectively, further supporting the conclusion that these antisera include antibodies that recognize the common ring structure element.

The binding of *R*-MVL (**2**) was considerably weaker than that of *R*,*S*-MVL, suggesting that the *S* enantiomer of the racemic haptens **10** and **11** was more antigenic in the rabbits. This result perhaps can be explained as an example of an unnatural enantiomer being inherently more immunogenic than the biologically relevant enantiomer, a phenomenon which has been noted in the case of enantiomeric amino acids.[49,50]

The present study has shown that antibodies recognizing *R*-MVL at lowered pH can be generated by use of a hapten structurally resembling *R*-MVL, thus demonstrating that an immunochemical approach to the accurate quantitation of mevalonic acid in biological

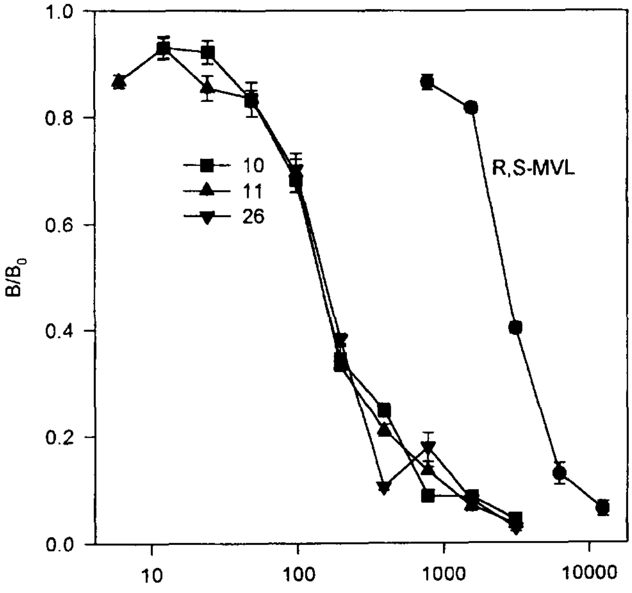


Figure 1. Competitive immunoassay for haptens **10**, **11** and **26** and *R*,*S*-mevalonolactone. Solutions of **10**, **11** and **26** and *R*,*S*-mevalonolactone compete with immobilized hapten **11**-KLH for binding at pH 4.0 to antibodies generated by immunization of rabbits with hapten **11**-KLH. Binding was measured as described in the Experimental section with a solid phase immunossay. IC_{50} for each hapten is defined as the amount of hapten required to inhibit the binding of a fixed amount of antibody by 50%.

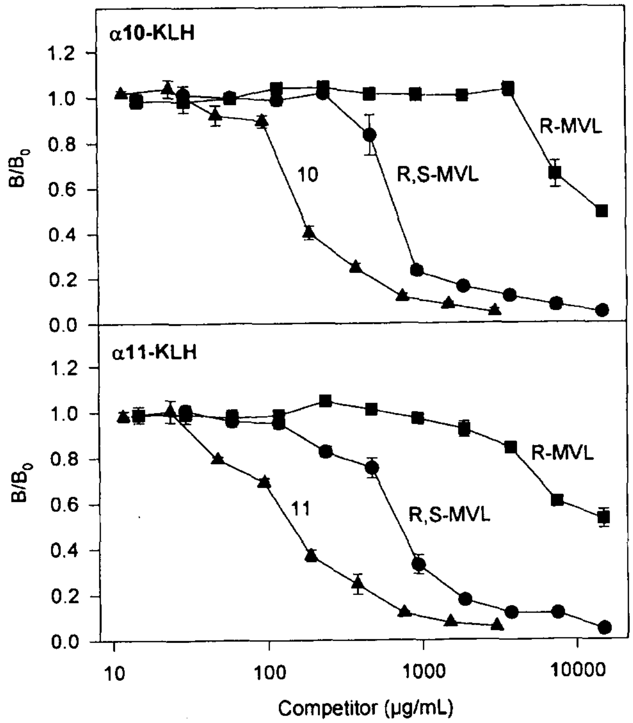


Figure 2. Competitive immunoassays with antisera generated by immunization with **10**-KLH (α**10**-KLH) and **11**-KLH (α**11**-KLH). Solutions of haptens **10** (or **11**), *R,S*-mevalonolactone and *R*-mevalonolactone (**2**) compete with immobilized hapten **10**-KLH or hapten **11**-KLH for binding to both rabbit antisera. Experimental details as described in the legend to Figure 1.

samples should be feasible. Further investigations will be required to generate antibodies with much higher affinity for the natural *R* enantiomer of mevalonolactone, ones which can detect the small, physiologically relevant concentrations in biological fluids (e.g., 100–200 nM in plasma). Strategies to generate such antibodies could include use of only *R* rather than racemic haptens for immunization, preparation of *R*-specific monoclonal antibodies, immunization of animals with very low concentrations of hapten conjugates, careful selection for antibodies of the highest affinity and improvement in sensitivity of detection of the antigen–antibody interaction.

Experimental

Chemical syntheses

General. All melting points and boiling points are uncorrected. Melting points were determined in a Thomas-Hoover capillary melting point apparatus using an Omega digital thermometer. Infrared (IR) spectra were recorded on a Perkin–Elmer 599 spectrometer and referenced to the 1601 cm^{-1} band of polystyrene. Liquid samples were analyzed as thin films on NaCl plates and solid samples were analyzed as transparent KBr disks. The ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) spectra were recorded on a Varian XL-300 (300 MHz) spectrometer using $CDCl_3$ as solvent, with exception of one ^{1}H NMR spectrum of **26** run on a Varian UNITYplus 500 MHz NMR spectrometer in benzene. Chemical shifts are reported in ppm downfield from tetramethylsilane. Elemental analyses were carried out by Atlantic Microlab, Inc., Atlanta, GA. Analytical thin-layer chromatography (TLC) was performed on EM silica gel 60 F254 plates from EM Science and visualization was accomplished with 254-nm light or by spraying with *p*-anisaldehyde–sulfuric acid–ethanol.[51] Flash chromatography was performed in the manner of Still[52] with EM Science silica gel 60 (230–400 mesh). The relative ratios in mixed chromatography solvents refers to the volume:volume ratio. High-resolution mass spectra (HRMS) were determined by Dr Gary Siuzdak at the Scripps Research Institute. Low resolution mass spectra were determined by Joanne Purdy at Dartmouth College on a Finnegan 4023 instrument at 35 eV using a solid probe.

Reactions requiring anhydrous conditions were performed in glassware that had been flame-dried and then allowed to cool in a desiccator. The term "under N_2" refers to maintenance of a positive pressure of nitrogen gas over the reaction mixture. Solvents were purified as follows: reagent grade hexane was distilled; tetrahydrofuran (THF) was distilled from sodium with benzophenone indicator; triethylamine was distilled and stored over KOH; methylene chloride and ether were distilled from $LiAlH_4$. All other commercially available reagents were obtained as reagent grade from Aldrich and used without further purification. Brine refers to a saturated aqueous NaCl solution.

Ethyl-(6-methyl-7-oxabicyclo[4.1.0]hept-2E-ylidene)-acetate (6) and ethyl-(6-methyl-7-oxabicyclo[4.1.0]hept-2Z-ylidene)-acetate (7). According to a modification of the procedure of Baggiolini et al.,[38] to a suspension of 3.3 g (0.14 mol) of sodium hydride (60% oil dispersion, washed with pentane) in dry THF (150 mL) at 0 °C under N_2 was added 30.9 g (0.14 mol) of triethyl phosphonoacetate dropwise. After the initial hydrogen evolution subsided, the resulting mixture was allowed to warm to room temperature, stirred for 1 h, and 14.5 g (0.15 mol) of **5**, prepared by the procedure of Wasson and House[37] from 3-methylcyclohex-2-en-1-one (**12**) prepared by the procedure of Cronyn and Riesser,[53] was added dropwise. The resulting mixture was stirred for an additional 12 h, then concentrated under reduced pressure, and the residual liquid was partitioned between 200 mL of ether and 200 mL of saturated aqueous sodium bicarbonate. The aqueous layer was washed with ether (3 × 200 mL) and the combined organic layers were washed with brine (50 mL), dried ($MgSO_4$), filtered and evaporated to afford a clear oil, which was distilled to afford 20.2 g (90%) of a mixture of **6** and **7**, (bp 90–92 °C (0.5 mmHg)), which ^{1}H NMR analysis indicated to be a 1:1 mixture of **6** and **7**. These were separated by repeated chromatography on silica gel (19:1, hexane:ether). For **6**, R_f = 0.22 (9:1, hexane:ether), an analytical sample was prepared by Kugelrohr distillation: bp 90–95 °C (0.45 mm Hg); IR (film) 3000, 2960, 1730, 1655, 1390, 1260, 1170, 1050, 890, 840 cm^{-1}; ^{1}H NMR 1.28 (3H, t, J = 7 Hz), 1.40 (3H, s), 1.30–1.78 (3H, m), 1.93–2.05 (1H, m), 2.30–2.45 (1H, m), 2.90–3.04 (1H, m), 3.12 (1H, s), 4.16 (2H, q, J = 7 Hz), 5.97 (1H, s); ^{13}C NMR δ 14.1, 17.7, 22.5, 25.0, 28.8, 59.6, 59.8, 62.4, 120.4, 154.7, 165.3. Anal. calcd for $C_{11}H_{16}O_3$: C, 67.31; H, 8.22. Found: C, 67.38; H, 8.26. For **7**, R_f = 0.20 (9:1, hexane:ether), an analytical sample was prepared by Kugelrohr distillation: bp 90–95 °C (0.45 mm Hg); IR (film) 3005, 2970, 2900, 1730, 1660, 1390, 1260, 1210, 1170, 935, 895 cm^{-1}; ^{1}H NMR δ 1.26 (3H, t, J = 7 Hz), 1.38 (3H, s), 1.30–1.79 (3H, m), 1.96–2.05 (2H, m), 2.27–2.39 (1H, m), 4.16 (2H, q, J = 7 Hz), 4.53 (1H, s), 5.90 (1H, s); ^{13}C NMR δ 14.2, 20.1, 23.5, 29.0, 30.5, 56.5, 60.0, 120.8, 155.1, 165.9. Anal. calcd for $C_{11}H_{16}O_3$: C, 67.31; H, 8.22. Found: C, 67.23; H, 8.23.

(1-Hydroxy-1-methylcyclohexyl-3E-ylidene)-ethanol (8). To a suspension of 140 mg (3.67 mmol) of $LiAlH_4$ in dry ether (30 mL) was added dropwise a solution of 300 mg (1.53 mmol) of **6** in dry ether (5 mL). The resulting mixture was stirred for 12 h and quenched initially with 1 g of sodium sulfate decahydrate and then 0.5 mL of water. The mixture was filtered and the salts were washed with ethyl acetate (50 mL). The combined organic layers were dried ($MgSO_4$), filtered, and evaporated to afford 287 mg of a white semisolid, which was recrystallized twice from ether to afford 156 mg (65%) of **8**, mp 59.0–60.5 °C, as a white solid. Repeated recrystallization from ether gave an analytical sample: mp 62.0–63.0 °C; IR (KBr) 3350, 2990, 2950, 2900, 2850, 1685, 1460, 1280, 1010, 995 cm^{-1}; ^{1}H NMR δ 1.23 (3H, s), 1.35–1.70 (6H, m), 1.82–1.95 (1H, m), 2.13 (1H, d, J = 13 Hz), 2.21 (1H, d, J = 13 Hz), 2.37–2.46 (1H, m), 4.17 (2H, d, J = 7 Hz), 5.44 (1H, t, J = 7 Hz); ^{13}C NMR δ 23.1, 27.8, 29.2, 38.9, 50.0, 58.4, 71.4, 124.1, 140.4. Anal. calcd for $C_9H_{16}O_2$: C, 69.19; H, 10.32. Found: C, 69.15; H, 10.37.

(1-Hydroxy-1-methylcyclohexyl-3Z-ylidene)-ethanol (9). To a suspension of 70.0 mg (1.84 mmol) of $LiAlH_4$ in dry ether (20 mL) was added dropwise a solution of 150 mg (0.76 mmol) of **7** in dry ether (5.0 mL). The resulting mixture was stirred for 12 h and quenched initially with 0.5 g of sodium sulfate decahydrate and then 0.25 mL of water. The same workup as for **8** gave 134 mg of white semisolid, which was recrystallized twice from ether to afford 75.0 mg (63%) of **9**, mp 52.0–53.0 °C. Repeated recrystallization from ether gave an analytical sample: mp 52.5–53.5 °C; IR (KBr) 3320, 2950, 2900, 2840, 1680, 1450, 1280, 1010, 995 cm^{-1}; ^{1}H NMR δ 1.27 (3H, s), 1.46–1.75 (5H, m), 1.90–2.22 (4H, m), 2.47–2.54 (1H, m), 3.95–4.23 (2H, m), 5.70 (1H, m); ^{13}C NMR δ 23.9, 29.7, 35.9, 39.0, 42.0, 57.7, 71.4, 123.7, 141.9. Anal. calcd for $C_9H_{16}O_2$: C, 69.19; H, 10.32. Found C, 69.09; H, 10.38.

3-Ethoxycarbonyl-(1-hydroxy-1-methylcyclohexyl-3E-ylidene)-propanoic acid (10). To a solution of 143 mg (0.91 mmol) of **8** and 308 mg (3.09 mmol) of succinic anhydride in dry THF (60 mL) was added 4.7 mL of dry triethylamine rapidly. The resulting mixture was heated at reflux for 5 days, allowed to cool to room temperature, then concentrated under reduced pressure, and the residual yellow oil was partitioned between 30 mL of ethyl acetate and 3 mL of 3 N aqueous HCl which had been saturated with NaCl. The aqueous layer was washed with ethyl acetate (3 × 15 mL) and the combined organic layers were washed with water (5 mL), brine (5 mL), dried ($MgSO_4$), filtered, and evaporated to afford 400 mg of yellow semisolid to which dry methylene chloride (20 mL) was added. The mixture was filtered and the filtrate was evaporated to afford 296 mg of pale-yellow oil, which was partitioned between 3 mL of methylene chloride and 3 mL of water. The resulting mixture was basified with solid sodium bicarbonate to pH 8. The aqueous layer was separated and added to 3 mL of methylene chloride. The resulting mixture was acidified to pH 4 with 3 N aqueous HCl which had been saturated with NaCl. The aqueous layer was washed with methylene chloride (7 × 2 mL) and the organic layers were combined, dried ($MgSO_4$), filtered, and evaporated to afford 235 mg of clear oil, which was dissolved in ethyl acetate (10 mL) and filtered through a florisil pad. The filtrate was evaporated to afford 174 mg (75%) of **10** as a clear oil: IR (film) 3000 (b), 2930, 1740, 1720, 1380, 1175, 1125, 975, 925 cm^{-1}; ^{1}H NMR δ 1.20 (3H, s), 1.45–1.90 (3H, m), 2.10–2.20 (2H, m), 2.40–2.67 (5H, m), 4.52 (1H, dd, J = 7, 12 Hz), 4.69 (1H, dd, J = 8, 12 Hz), 5.30–5.37 (1H, m), 6.71 (2H, bs); ^{13}C NMR d 22.9, 28.0, 28.7, 29.0, 29.1, 36.8, 49.8, 60.5, 72.0, 118.8, 143.2, 172.2, 176.5. Anal. calcd for $C_{13}H_{20}O_4$: C, 60.92; H, 7.87. Found: C, 60.65; H, 7.94.

3-Ethoxycarbonyl-(1-hydroxy-1-methylcyclohexyl-3Z-ylidene)-propanoic acid (11). To a solution of 85 mg (0.54 mmol) of **9** and 184 mg (1.84 mmol) of succinic anhydride in dry THF (40 mL) was added 2.8 mL of dry triethylamine rapidly. The resulting mixture was heated at reflux for 5 days, and then processed as in the preparation of **10** to afford 119 mg of yellow semisolid to which 20 mL of dry methylene chloride was added. The mixture was filtered and the filtrate was evaporated to afford 115 mg of pale-yellow oil, which was partitioned between 2 mL of methylene chloride and 2 mL of water. The resulting mixture was processed as in the preparation of **10** to afford 100 mg of clear oil, which was dissolved in ethyl acetate (10 mL) and filtered through a florisil pad. The filtrate was evaporated to afford 70 mg (50%) of **11** as a clear oil: IR (film) 3000 (b), 2930, 1740, 1720, 1330, 1160, 915 cm^{-1}; ^{1}H NMR δ 1.25 (3H, s), 1.43–1.73 (4H, m), 1.94–2.21 (3H, m), 2.48–2.69 (5H, m), 4.47 (1H, dd, J = 7, 12 Hz), 4.73 (1H, dd, J = 8, 12 Hz), 5.47 (1H, m); ^{13}C NMR δ 23.4, 29.0, 29.1, 29.4, 35.7, 38.7, 42.6, 60.7, 71.5, 118.5, 143.9, 172.5, 176.8. Anal. calcd for $C_{13}H_{20}O_4$: C, 60.92; H, 7.87. Found: C, 60.70; H, 7.90.

3-Methyl-6-(2-propenyl)-2-cyclohexen-1-one (13). To a solution of 5.6 mL (39.9 mmol) of dry diisopropylamine in dry THF (150 mL) at 0 °C under N_2 was added 25.0 mL (39.9 mmol) of *n*-butyllithium (1.60 M in hexane) dropwise via syringe. The resulting suspension was stirred for 15 min at 0 °C and then cooled to −78 °C and stirred an additional 15 min. A solution of 4.00 g (36.3 mmol) of **12** (**15**) in dry THF (20 mL) was added dropwise via syringe. The mixture was stirred at −78 °C for 30 min and then 7.00 g (57.9 mmol) of allyl bromide was added dropwise via syringe. The resulting mixture warmed to room temperature overnight while being stirred. The light-yellow solution was concentrated under reduced pressure to afford 14.8 g of yellow oil, which was diluted with ether (200 mL), washed with water (2 × 25 mL) and brine (25 mL), dried ($MgSO_4$), filtered, and evaporated to afford 5.99 g of light-yellow oil, which was distilled to afford 4.38 g (80%) of **13** as a clear oil: bp 53–55 °C (0.25 mm Hg); IR (film) 2900, 1670, 1425, 1380, 910 cm^{-1} (lit.[44] IR (CCl_4) 2980, 2920, 2860, 1680, 1640, 1546, 1250, 1220, 1000, 980, 912, 810, 745 cm^{-1}); ^{1}H NMR δ 1.55–1.70 (1H, m), 1.88 (3H, s), 1.95–2.30 (5H, m), 2.60–2.70 (1H, m), 4.95–5.05 (2H, m), 5.63–5.80 (1H, m), 5.78 (1H, s) (lit.[30] ^{1}H NMR (90 MHz, $CDCl_3$) δ 1.33–2.87 (10H, m), 4.80–5.16 (2H, m), 5.50–6.03 (2H, m)); ^{13}C NMR δ 24.0, 27.1, 30.2, 33.6, 44.8, 116.4, 126.1, 136.2, 161.6, 201.5. Anal. calcd for $C_{10}H_{14}O$: C, 79.96; H, 9.39. Found: C, 79.78; H, 9.36.

6-Methyl-3-(2-propenyl)-2-oxo-7-oxabicyclo[4.1.0]heptane (14) and 6α-methyl-3β-(2-propenyl)-2-oxo-7-oxabicyclo[4.1.0]heptane (15). According to a modification of the procedure of Solladie,[54] a solution of 4.80 g (31.9 mmol) of **13** and 6.5 mL of 30% hydrogen peroxide in methanol (120 mL) and water (60 mL) was cooled to 0 °C and 2.5 mL of saturated aqueous potassium carbonate was added dropwise. The resulting mixture was stirred at 0 °C for 2 h and then at room temperature for 1 h, poured into water (100 mL), and the aqueous layer was washed with methylene chloride (8 × 50 mL). The combined organic layers were dried ($MgSO_4$), filtered, and evaporated to afford 5.22 g of clear oil. Purification of a 1.29-g portion of this crude product by chromatography on silica gel (1:1, hexane:ethyl acetate) afforded 1.11 g (88%) of **14** and **15**, in a 3:2 ratio as indicated by ^{13}C NMR, as a clear viscous oil: IR (film) 2920, 1710, 1440, 1400, 1200, 910 cm^{-1}; ^{1}H NMR δ 1.34 (3H, s), 1.36 (3H, s), 1.50–2.65 (14H, m), 2.97 (1H, s), 3.02 (1H, s), 4.85–5.00 (4H, m), 5.50–5.70 (2H, m); ^{13}C NMR δ 21.2, 21.6, 22.9, 26.8, 26.8, 28.5, 33.3, 34.8, 41.7, 45.6, 61.0, 61.4, 62.1, 65.5, 116.6, 116.8, 135.2, 135.6, 206.6, 208.8. Anal. calcd for $C_{10}H_{14}O_2$: C, 72.26; H, 8.42. Found: C, 72.03; H, 8.44.

1α-Methyl-4β-(2-propenyl)-1β,3β-cyclohexanediol (16), 1α-methyl-4β-(2-propenyl)-1β,3α-cyclohexanediol (17), 1β-methyl-4β-(2-propenyl)-1α,3β-cyclohexanediol (18), and 1β-methyl-4β-(2-propenyl)-1α,3α-cyclohexanediol (19). To a suspension of 255 mg (6.74 mmol) of $LiAlH_4$ in dry ether (50 mL) was added dropwise a solution of 450 mg (2.79 mmol) of the mixture of **14** and **15** in dry ether (10 mL). The resulting mixture was stirred for 3 h and quenched initially with 0.5 g of sodium sulfate decahydrate and then with 1.0 mL of water. The mixture was filtered and the salts were washed with ethyl acetate (25 mL). The combined organic layers were dried ($MgSO_4$), filtered and evaporated to afford 464 mg of an approximately 1:2:1:1 mixture of **16**, **17**, **18** and **19**, as indicated by ^{13}C NMR. This mixture was partially separated and purified by chromatography on silica gel (ethyl acetate), to afford 57 mg (12%) of **16** as a clear oil, 63 mg (13%) of **17**, mp 113.5–114.5 °C, and 81 mg (17%) of a 1:1 mixture of **18** and **19** as a clear oil. In subsequent experiments on a larger scale, approximately 85% of the total amount of **17** present crystallized from the crude product upon standing.

For **16**, TLC R_f = 0.49 (ethyl acetate); IR (film) 3350 (b), 2940, 1650, 1450, 1160, 910 cm^{-1}; ^{1}H NMR δ 1.21 (3H, s), 1.20–1.40 (4H, m), 1.50–1.71 (2H, m), 1.85–2.21 (3H, m), 3.85–3.90 (1H, m), 3.95 (1H, s), 4,00 (1H, s), 4.90–5.07 (2H, m), 5.69–5.83 (1H, m); ^{1}H NMR ($CDCl_3$ + 2 drops D_2O) δ 1.21 (3H, s), 1.20–1.40 (4H, m), 1.50–1.71 (2H, m), 1.85–2.21 (3H, m), 3.85–3.90 (1H, m), 4.90–5.07 (2H, m), 5.69–5.83 (1H, m); ^{13}C NMR δ 21.9 (u), 30.7 (d), 37.1 (u), 38.7 (u), 41.0 (d), 43.0 (u), 69.6 (d), 70.6 (u), 115.8 (u), 137.1 (d). Anal. calcd for $C_{10}H_{18}O_2$: C, 70.55; H, 10.66. Found: C. 70.43; H, 10.56.

For **17**, TLC R_f = 0.33 (ethyl acetate); recrystallization from ether gave an analytical sample: mp 114.0–114.5 °C; IR (KBr) 3340 (b), 2920, 1650, 1440, 1360, 1350, 1250, 1030, 900 cm^{-1}; ^{1}H NMR δ 1.24 (3H, s), 1.25–1.28 (3H, m), 1.50–1.67 (2H, m), 1.80–2.05 (5H, m), 2.42–2.55 (1H, m), 3.57–3.76 (1H, dt, J = 4.1, 10.2 Hz), 4.98–5.05 (1H, dd, J = 1.3, 11.1 Hz), 5.02–5.11 (1H, dd, J = 1.3, 18.9), 5.78–5.93 (1H, ddt, J = 7.0, 10.0, 17.1 Hz); ^{1}H NMR ($CDCl_3$ + 2 drops D_2O) δ 1.24 (3H, s), 1.25–1.28 (3H, m), 1.50–1.67 (2H, m), 1.80–2.05 (5H, m), 2.42–2.55 (1H, m), 3.57–3.76 (1H, t, J = 4.1, 10.2 Hz), 4.98–5.05 (1H, dd, J = 1.3, 11.1 Hz), 5.02–5.11 (1H, dd, J = 1.3, 18.9), 5.78–5.93 (1H, ddt, J = 7.0, 10.0, 17.1 Hz); ^{13}C NMR δ 25.6 (u), 31.5 (d), 37.1 (u), 38.1 (u), 44.3 (d), 47.5 (u), 71.1 (u), 71.5 (u), 116.2 (u), 137.4 (d). Anal. calcd for $C_{10}H_{18}O_2$: C, 70.55; H, 10.66. Found: C, 70.63; H, 10.68.

For **18** and **19**, TLC R_f = 0.31 (ethyl acetate); ^{1}H NMR δ 1.13 (3H, s), 1.23 (3H, s), 1.30–1.95 (m), 2.12–2.25 (1H, m), 3.47–3.56 (1H, m), 3.98–4.06 (1H, m), 4.90–5.03 (4H, m), 5.60–5.80 (2H, m); ^{1}H NMR ($CDCl_3$ + 2 drops D_2O) δ 1.13 (3H, s), 1.23 (3H, s), 1.30–1.95 (m), 2.12-2.25 (1H, m), 3.41-3.49 (1H, m), 3.96-4.04 (1H, m), 4.90–5.03 (4H, m), 5.60–5.80 (2H, m); ^{1}H NMR (D_2O) δ 1.15 (3H, s), 1.23 (3H, s), 1.30–1.95 (1H, m), 2.12–2.25 (1H, m), 2.35–2.48 (1H, m), 3.28–3.38 (1H, dt, J = 4.2, 10.4 H), 3.99–4.06 (1H, m), 4.90–5.03 (2H, m), 5.60–5.80 (1H, m); ^{13}C NMR δ 22.6, 22.9, 28.7, 30.6, 34.5, 35.5, 35.5, 35.9, 39.3, 41.3, 43.1, 43.4, 68.5, 71.0, 71.0, 71.3, 115.6, 116.0, 137.0, 137.8. Anal. calcd for $C_{10}H_{18}O_2$: C, 70.55; H, 10.66. Found: C, 69.05; H, 10.64. FAB HRMS calcd for $C_{10}H_{18}O_2Na$ (M + Na^+) 193.1205, found 193.1205.

Oxidation of 17 to 5α-methyl-2β-(2-propenyl)-5β-hydroxycyclohexan-1-one (20). In the manner of Griffith et al.,[46] to a mixture of 100 mg (0.59 mmol) of **17**, 45 mg (0.34 mmol) of *N*-methylmorpholine *N*-oxide monohydrate (NMNO) and 60 mg of crushed 4 Å molecular sieves in dry methylene chloride (10 mL), which had been stirred for 15 min, was added 10 mg (0.03 mmol) of tetra-*n*-propylammonium perruthenate in one portion. The resulting mixture was stirred for 12 h, filtered through a plug of silica gel with ethyl acetate, and concentrated to afford 100 mg of **20** as a clear oil, contaminated by ca. 15% NMNO. This mixture was not further purified. For **20**, ^{1}H NMR δ 1.27 (3H, s), 1.61–1.95 (5H, m), 2.15–2.50 (4H, m), 4.85–4.98 (2H, m), 5.60–5.78 (1H, m); ^{13}C NMR δ 27.7, 30.5, 33.0, 37.2, 48.8, 54.3, 74.4, 115.9, 136.1, 211.2.

Oxidation of 16 to 5α-methyl-2β-(2-propenyl)-5β-hydroxycyclohexan-1-one (20). By the same procedure, 215 mg (1.26 mmol) of **16**, 234 mg (1.73 mmol) of NMNO, 90 mg of crushed 4 Å molecular sieves in dry methylene chloride (20 mL), and 10 mg (0.03 mmol) of tetra-*n*-propylammonium perruthenate afforded 156 mg of a clear, oily 3:1 mixture of **20** and **16**, as indicated by ^{13}C NMR.

Oxidation of 18 and 19 to 5β-methyl-2β-(2-propenyl)-5β-hydroxycyclohexan-1-one (21). By the same procedure, 187 mg (1.10 mmol) of the mixture of **18** and **19**, 204 mg (1.51 mmol) of NMNO, 70 mg of crushed 4 Å molecular sieves in dry methylene chloride (10 mL), and 20 mg (0.06 mmol) of tetra-*n*-propylammonium perruthenate afforded 274 mg of **21** as a clear oil, which was contaminated with ca. 50% NMNO. For **21**, ^{13}C NMR δ 26.5, 27.1, 33.3, 37.2, 48.2, 54.8, 73.0, 116.1, 135.5, 210.2.

Reduction of 20 derived from 17 to a mixture of 16 and 17. To a suspension of 56 mg (1.5 mmol) of LiAlH$_4$ in dry ether (10 mL) was added dropwise a solution of 100 mg (0.59 mmol) of crude **20** prepared from **17** as described above, in dry ether (3 mL). The resulting mixture was stirred for 1 h and quenched initially with 1.0 g of sodium sulfate decahydrate and then with 0.5 mL of water. The mixture was filtered and the salts were washed with chloroform (15 mL). The combined organic layers were dried (MgSO$_4$), filtered, and evaporated to afford 100 mg of a 4:1 mixture of 16 and **17**, as indicated by ^{13}C NMR, as a cloudy viscous oil: ^{13}C NMR δ 21.9, 25.6, 30.5, 31.2, 36.7, 37.0, 38.1, 38.6, 40.9, 42.9, 44.0, 47.4, 69.4, 70.6, 70.6, 71.2, 115.6, 115.8, 137.1, 137.2.

Reduction of 20 derived from 16 to a mixture of 16 and 17. In exactly the same manner, 155 mg (0.92 mmol) of crude **20**, prepared from **16** as described above, afforded 150 mg of a 4:1 mixture of 16 and **17**, as indicated by ^{13}C NMR, as a cloudy viscous oil: ^{13}C NMR δ 21.9, 25.4, 30.7, 31.3, 36.8, 37.1, 37.9, 38.7, 40.9, 43.0, 44.1, 47.5, 69.5, 70.7, 70.8, 71.2, 115.7, 115.9, 137.2, 137.3.

Reduction of 21 derived from 18 and 19 to a mixture of 18 and 19. In exactly the same manner, 180 mg (1.07 mmol) of **21**, prepared as described above afforded 171 mg of a 1:1 mixture of **18** and **19**, as indicated by ^{13}C NMR, as a cloudy viscous oil: ^{13}C NMR δ 22.6, 22.9, 28.6, 30.6, 34.5, 35.5, 35.5, 35.8, 39.3, 41.2, 43.3, 43.4, 68.4, 70.8, 71.1, 71.3, 115.6, 115.9, 136.9, 137.8.

1α-Methyl-4β-(2-propenyl)-1β,3β-cyclohexanecarbonate (22). In the manner of Kutney and Ratcliffe,[55] to a solution of 150 mg (0.88 mmol) of **16** in dry benzene (20 mL) was added 100 mg (0.62 mmol) of *N,N′*-carbonyldiimidazole in one portion. The resulting solution was heated at reflux for 1 h, after which an additional 100 mg (0.62 mmol) of *N,N′*-carbonyldiimidazole was added, followed by a further addition of 371 mg (2.90 mmol) of *N,N′*-carbonyldiimidazole in small portions to the refluxing reaction mixture over 5 h. The resulting mixture was heated at reflux for an additional 12 h and concentrated under reduced pressure to afford an oily white solid. The residue was dissolved in chloroform (30 mL) and washed with water (20 mL). The aqueous layer was washed with chloroform (4 × 25 mL) and the combined organic layers were dried (MgSO$_4$), filtered, and evaporated to afford 833 mg of clear oil, which was purified by chromatography on silica gel (7:3, ethyl acetate:hexane) to afford 25 mg (14%) of **22**, mp 91.0–93.5 °C. Recrystallization from ether gave an analytical sample: mp 95.5–96.0 °C; TLC R_f = 0.50 (7:3, ethyl acetate:hexane) IR (KBr) 2750, 1700, 1390, 1220, 1140, 1090 cm^{-1}; ^{1}H NMR δ 1.41 (3H, s), 1.45–1.65 (3H, m), 1.70–1.80 (2H, m), 1.99–2.31 (4H, m), 4.57–4.62 (1H, m), 5.03–5.15 (2H, m), 5.68–5.82 (1H, m); ^{13}C NMR δ 23.1, 27.7, 35.1, 36.5, 36.5, 40.2, 80.5, 117.5, 135.2, 150.1. Anal. calcd for $C_{11}H_{16}O_3$: C, 67.32; H, 8.22. Found: C, 67.10; H, 8.18.

1α-Methyl-4β-(2-propanal)-1β,3α-cyclohexanediol (23) and 1α-methylcyclohexan-1β-ol-3γ-lactol (24). Through a solution of 150 mg (1.00 mmol) of **17** in dry methylene chloride (10 mL) and dry methanol (1 mL), cooled to −78 °C, was bubbled ozone until a blue color formed after 10 min. The ozone was replaced with argon and after 5 min 300 mg (1.15 mmol) of triphenylphosphine was added in one portion. The resulting mixture was warmed to room temperature while being stirred overnight, and then concentrated under reduced pressure to afford 480 mg of clear oil which was purified by chromatography on silica gel (5:60:35, acetic acid:ethyl acetate:ether). Acetic acid was removed azeotropically with heptane from appropriate fractions to afford 131 mg (86%) of a 5:1 mixture of **23** and **24**, as indicated by ^{13}C NMR, as a clear oil: IR (film) 3300 (b), 2900, 1713, 1450, 1373, 1039 cm^{-1}: ^{1}H NMR δ 1.22 (3H, s), 1.26 (3H, s), 1.20–2.40 (m), 2.65–2.75 (m), 3.50–3.60 (m), 3.76–3.90 (m), 5.40–5.55 (m), 9.75 (1H, s); for **23**, ^{13}C NMR δ 26.8, 31.4, 37.7, 39.9, 47.7, 48.0, 70.7, 71.5, 203.7. FAB HRMS calcd for $C_9H_{16}O_3Na$ (M + Na$^+$) 195.0997, found 195.0993.

4β-(Carboxy-2Z-hexylidene)-1α-methyl-1β,3α-cyclohexanediol (25). In the manner of Maryanoff,[56] to a suspension of 2.06 g (4.64 mmol) of (4-carboxybutyl)-triphenylphosphonium bromide in dry THF (20 mL) was added 18.6 mL (9.29 mmol) of potassium bis(trimethylsilyl)amide (0.50 M in toluene) dropwise. The resulting mixture was stirred for 15 min and then a solution of 200 mg (1.16 mmol) of the mixture of **23** and **24** in dry THF (10 mL) was added dropwise to the bright orange solution. The resulting tan mixture was stirred for 2 h at room temperature. The reaction mixture was partitioned between water (50 mL) and ether (50 mL) and the organic layer was washed with water (30 mL). The combined aqueous layers were washed with ethyl acetate (50 mL), acidified with 1 M aqueous H_2SO_4, and washed with ethyl acetate (4 × 50 mL). The combined organic layers were washed with water (20 mL), brine (20 mL), dried ($MgSO_4$), filtered, and evaporated to afford 832 mg of a light-yellow oily mixture of triphenylphosphine oxide, (4-carboxybutyl)-triphenylphosphonium bromide and **25**. For **25**: ^{13}C NMR δ 25.7, 26.2, 28.6, 29.4, 128.2, 128.3, 128.5, 128.6, 130.3, 130.5, 130.6, 131.7, 131.8, 131.9, 176.0. FAB HRMS calcd for $C_{14}H_{24}O_4$ ($M-H^+$) 255.1596, found 255.1586.

2-(Carboxy-2Z-hexylidene)-5α-methyl-1β-hydroxycyclohexan-1-one (26). In the manner of Griffith et al.,[46] to a mixture of 1.086 g of a crude product containing **25** as described above, 274 mg (1.76 mmol) of NMNO, and 0.050 g of crushed 4 Å molecular sieves in dry methylene chloride (15 mL) which had been stirred for 15 min, was added 0.025 g (0.07 mmol) of tetra-*n*-propylammonium perruthenate in one portion. The resulting mixture was stirred for 12 h, filtered through a plug of silica gel (chloroform), and concentrated to afford 1.10 g of dark-purple oil, which was purified by chromatography (3 times) on silica gel (95:5, ethyl acetate:acetic acid) to afford 0.058 g (20%) of **26**, mp 74.0–75.5 °C. Recyrstallization from ether gave an analytical sample: mp 78.5–79.5 °C; IR (KBr) 3420, 1706, 1691, 1234, 1164 cm^{-1}; ^{1}H NMR δ 1.32 (3H, s), 1.60–2.50 (15, m), 5.30–5.40 (2H, m), 6.90 (2H, bs); ^{1}H NMR (500 MHz, benzene) 5.32 (1H, m), 5.40 (1H, m). Simulation of this region of the spectrum with the gNMR program[57] using the experimental data file and initial coupling constants estimated from a DQCOSY spectrum indicated that the alkenyl protons have J = 10.6 Hz. ^{13}C NMR δ 24.4, 26.4, 26.5, 28.0, 30.6, 33.3, 37.3, 49.5, 54.5, 75.1, 128.1, 130.1, 178.7, 211.8. FAB HRMS calcd for $C_{14}H_{22}O_4Na$ ($M+Na^+$) 277.1416, Found 277.1421. Anal. calcd for $C_{14}H_{22}O_4$: C, 66.12; H, 8.72. Found: C, 64.55; H, 8.65.

Immunologic procedures

Preparation of hapten–KLH conjugates. Haptens **10** and **11** were coupled to keyhole limpet hemocyanin (KLH) (Pierce, 77100) according to the method of Cianciolo.[58] The haptens were dissolved in 1.84 mL of H_2O, pH 5.5, to 1 mM and added to 40 mg of 1-ethyl-3(3-diethylamine propyl)carbodiimide hydrochloride (Pierce, 22980). After 1.0 min, 0.16 mL of KLH in H_2O (25 mg/mL) was added dropwise with stirring. The reaction was allowed to continue for 6 h at room temperature and maintained at a pH of 5.0 with the addition of dilute HCl. The samples were adjusted to 1.0 M glycine by the addition of 150 mg of solid reagent, mixed and rotated for 17 h at 4 °C. The samples were dialyzed exhaustively against H_2O, then phosphate buffered saline (PBS) and stored at −70 °C.

Preparation of antibodies. New Zealand white rabbits were injected subcutaneously at multiple sites with a total of 135 mg of hapten **10**- or hapten **11**-KLH conjugate in the presence of complete Freund's adjuvant. Equal amounts of hapten conjugates were given as secondary injections in the presence of incomplete Freund's adjuvant on days 21 and 45. The sera were collected on day 60 and stored frozen at −20 °C.

Immunoassays. Assays were performed in triplicate in polystyrene 96-well microtiter plates (Dynatech Immulon 4). All incubations were performed at 24 °C on a rotating platform and the buffers and solutions used were obtained in a Pierce #91320 ELISA kit. The plates were coated with 0.05 mL of a hapten–KLH conjugate at 50 mg/mL in coating buffer, which contained 6.2 M $NaCO_3/NaHCO_3$, pH 9.4. They were blocked with bovine serum albumin (BSA) in Dulbecco's Modified PBS and washed with Dulbecco's PBS containing 0.2% Tween 20. Competitors (haptens **10**, **11** and **26**, and *R*,*S*-MVL from Sigma) and antibodies (0.025 mL of each in 2 mM $NaPO_4$, pH 4.0) were incubated together in the microtiter plate for 16 h. After washing, binding of the rabbit antibodies was detected by a 1-h incubation with 0.05 mL of alkaline phosphase-conjugated goat anti-rabbit heavy- and light-chain IgG. Development of color from the 4-nitrophenyl phosphate chromogenic substrate was recorded after 1 h at an absorbance of 405 mM on a Thermo Max Microplate Reader (Molecular Devices). Nonspecific binding was determined by replacing specific rabbit antisera with a normal rabbit serum. Data were expressed as the ratio B/Bo, where Bo is the absorbance of wells containing no competitor minus nonspecific binding and B is the absorbance of wells containing competitor minus nonspecific binding.

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References

1. Goldstein, J. L.; Brown, M. S. *Nature* **1990**, *343*, 425.

2. Casey, P. J. *J. Lipid Res.* **1992**, *33*, 1731.

3. Grünler, J.; Ericcson, J.; Dallner, G. *Biochim. Biophys. Acta* **1994**, *1212*, 259.

4. Singer, I. I.; Kawaka, D. W.; Kagagis, D. W.; Alberts, A.W.; Cho, J. S.; Huff, J. W.; Ness, G. C. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 5556.

5. Ma, P. T. S.; Gil, G.; Sudhof, T. C. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 8370.

6. Jakóbisiak, M.; Bruno, S.; Skierski, J. S.; Darynkievicz, Z. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 3628.

7. Keyomarsi, K.; Sandoval, L.; Pardee, V. B.; Pardee, A. B. *Cancer Res.* **1991**, *51*, 3602.

8. Perez-Sala, D.; Mollinedo, F. *Biochem. Biophys. Res. Commun.* **1994**, *199*, 1209.

9. Jones, K. D.; Cauldwell, W. T.; Hinton, D. R.; Su, Y.; He, S.; Amber, L.; Law, R. E. *Biochem. Biophys. Res. Commun.* **1994**, *205*, 1681.

10. Borner, M. M.; Myers, C. E.; Sator, O.; Sei, Y.; Tobo, T.; Tyrel, J. B.; Schneider, E. *Cancer Res.* **1995**, *55*, 2122.

11. cf. Endo, A.; Kurodon, M.; Tanazawa, K. *FEBS Lett.* **1976**, *72*, 323.

12. Yamamoto, A. V.; Sudo, H.; Endo, A. *Athero.* **1980**, *35*, 259.

13. Alberts, A. W.; Chen, J.; Kuron, G.; Hunt, V.; Hoffman, C.; Rothrock, J.; Lopez, M.; Joshua, H.; Harris, E.; Patchett, A.; Monaghan, R.; Currie, S.; Stopley, E.; Alberts-Schonberg, G.; Hensens, O.; Hirshfield, G.; Hoogsteen, K.; Liesch, J.; Springer, J. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 3957.

14. Sumi, S.; Beauchamp, R. D.; Townsend, C. M., Jr.; Uchido, T.; Murakami, M.; Rajaraman, S.; Ishizuka, J.; Thompson, J. C. *Gastroenterology* **1992**, *103*, 982.

15. Soma, M. R.; Pagliarini, P.; Butti, G.; Paoletti, R.; Paoletti, P.; Fumagalli, R. *Cancer Res.* **1992**, *52*, 4348.

16. Miller, A. C.; Kariho, K.; Myers, C. E.; Clark, E. P.; Samid, D. *Int. J. Cancer* **1993**, *53*, 302.

17. Larsson, O. *Glycobiology* **1993**, *3*, 475.

18. Newman, A.; Clutterback, R. D.; Powles, R. L.; Miller, J. L. *Leukemia* **1994**, *8*, 2023.

19. Sumi, S.; Beauchamp, R. D.; Townsend, C. M., Jr.; Uchida, T.; Murakami, M.; Rajaraman, S.; Ishizuka, J.; Thompson, J. C. *Gastro.* **1992**, *103*, 982.

20. Kopito, R. R.; Brunnengraber, H. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *77*, 5738.

21. Parker, T. S.; McNamara, D. J.; Brown, C. D.; Kolb, R.; Ahrens, E. H., Jr.; Alberts, A. W., Tobert, J.; Chen, J.; DeSchepper, P. J. *J. Clin. Invest.* **1984**, *74*, 795.

22. Illingworth, D. R.; Pappu, A. S.; Gregg, R. E. *Athero.* **1989**, *76*, 21.

23. Pappu, A. S.; Illingworth, D. R. *J. Lab. Clin. Med.* **1989**, *114*, 554.

24. Pappu, A. S.; Illingworth, D. R.; Bacon, S. *Metabolism* **1989**, *38*, 542.

25. Jenkins, D. J. A.; Khan, A.; Jenkins, A. L.; Illingworth, R.; Pappu, A. S.; Wolever, T. M. S.; Vuksar, V.; Buckley, G.; Rao, A. V.; Cunnane, S. C.; Brighenti, F.; Hawkins, M.; Abdolell, M.; Corey, P.; Patten, R.; Jose, R. G. *Metabolism* **1995**, *44*, 549.

26. Hohl, R. J.; Lewis, K.; Clamon, G.; Knapp, H. *J. Invest. Med.* **1995**, *43*, 478A.

27. Hagenfeldt, L.; Hellström, K. *Life Sci.* **1972**, *11*, Part II, 669.

28. Del Puppo, M.; Cighetti, G.; Galli Kienle, J.; de Angelis, L. *Biomed. Environ. Mass Spectrom.* **1989**, *18*, 174.

29. Scoppola, A.; Maher, V. M. G.; Thompson, G. R.; Rendell, N. B.; Taylow, G. W. *J. Lipid. Res.* **1991**, *32*, 1057.

30. Lindenthal, B.; von Bergmann, K. *Biol. Mass. Spectrom.* **1994**, *d23*, 445.

31. Ishihama, Y.; Mano, N.; Oda, Y.; Hiyoshi, H.; Ikuta, H.; Yoshida, Y. *Rapid Commun. Mass Spectrom.* **1994**, *8*, 377.

32. Gondoh, K.; Itoh, S.; Tsukada, Y. *Rinsho Kagaku* **1995**, *24*, 89.

33. Popjak, G.; Boehm, G.; Parker, T. S.; Edmond, J.; Edwards, P. A.; Fogelman, A. M. *J. Lipid. Res.* **1979**, *20*, 716.

34. Curtiss, L. K.; Krueger, R. G. *Immunochemistry* **1975**, *12*, 949.

35. Bushweller, J. H.; Anzalone, L.; Spencer, T. A. *Synth. Comm.* **1989**, *19*, 745.

36. Ray, N. C.; Raveendranath, P. C.; Spencer, T. A. *Tetrahedron* **1992**, *48*, 9427.

37. Wasson, R. L.; House, H. O. *Organic Syntheses* **1963**, *CV IV*, 552.

38. Baggiolini, E. G.; Iacobelli, J. A.; Hennessey, B. M.; Batcho, A. D.; Sereno, J. F.; Uskokovic, M. R. *J. Org. Chem.* **1986**, *51*, 3098.

39. Bensel, N.; Marshall, H.; Weyerstahl, P. *Tetrahedron Lett.* **1976**, 2293.

40. Osuka, A.; Taka-Oka, K.; Suzuki, H. *Chem. Lett.* **1984**, 271.

41. Pfau, M.; Ughetto-Monfrin, J.; Joulain, D. *Bull. Soc. Chim. France* **1979**, *II*, 627.

42. Marino, J. P.; Jaen, J. C. *J. Am. Chem. Soc.* **1982**, *104*, 3165.

43. Kuwajima, I.; Nakamura, E.; Shimizu, M. *J. Am. Chem. Soc.* **1982**, *104*, 1025.

44. Stanton, S. A.; Felman, S. W.; Parkhurst, C. S.; Godleski, S. A. *J. Am. Chem. Soc.* **1983**, *105*, 1964.

45. Podraza, K. F. *Org. Prep. Proc. Int.* **1991**, *23*, 217.

46. Griffith, E. P.; Ley, S. V.; Whitcombe, G. P.; White, A. D. *J. Chem. Soc. Chem. Comm.* **1987**, 1625.

47. Nader, F. W.; Heinrich, W.; Baar-Schäfer, M.; Hangel, E. *Chem. Ber.* **1985**, *118*, 4314.

48. Vedejs, E.; Peterson, M. J. In *Topics in Stereochemistry*; Eliel, E. L.; Wilen, S. H., Eds.; John Wiley: New York, 1990; Vol 21, pp. 1–157; see Table 21, p. 108.

49. Schechter, I.; Sela, M. *Biochemistry* **1967**, *6*, 897.

50. Pollack, S. J.; Hsiun, P.; Schultz, P. G. *J. Am. Chem. Soc.* **1989**, *111*, 5961.

51. Gordon, A. J.; Ford, R. In *The Chemist's Companion. A Handbook of Practical Data, Techniques, and References*; John Wiley: New York, 1972; p. 379.

52. Still, W. C.; Kahn, M.; Mitra, A. *J. Org. Chem.* **1978**, *43*, 2923.

53. Cronyn, M. W.; Riesser, G. H. *J. Am. Chem. Soc.* **1953**, *75*, 1664.

54. Solladié, G.; Hutt, J. *J. Org. Chem.* **1987**, *52*, 3560.

55. Kutney, J. P.; Ratcliffe, A. H. *Synth. Comm.* **1975**, *5*, 47.

56. Maryanoff, B. E.; Duhl-Emswiler, B. A. *Tetrahedron Lett.* **1981**, *22*, 4185.

57. The gNMR Spectral Simulation program from Softshell International, Ltd., Grand Junction, CO.

58. Cianciolo, G. J.; Copeland, T. D.; Oroszlan, S.; Snyderman, R. *Science* **1985**, *230*, 453.

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