Synthesis of a Pyrazole Isostere of Pyrroles Formed by the Reaction of the ϵ-Amino Groups of Protein Lysyl Residues with Levuglandin E_2[1]

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A pyrazole isostere of pyrroles that are formed in the reaction of levuglandin E_2 (LGE_2) with proteins was prepared. The heterocycle was generated, together with a structural isomer, by condensation of a monoalkylhydrazine with a 1,3-diketone. Structures of the isomeric pyrazoles were established by NOE experiments. Coupling with proteins to provide antigens was achieved in the presence of alkene, carboxyl, and allylic hydroxyl functionality by reductive alkylation with an aldehyde group at the end of an *n*-alkyl tether to the 1-position of the pyrazole isostere.

Introduction

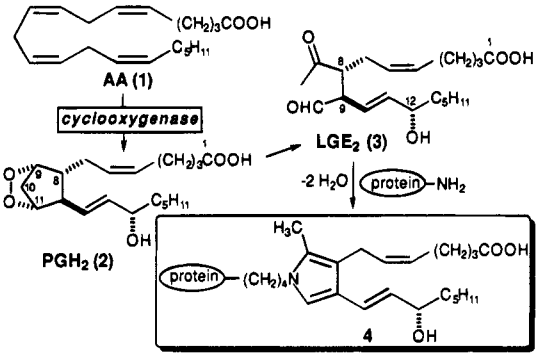
Levuglandin E_2 (LGE_2, **3**) is generated (Scheme 1) by a rearrangement of the prostaglandin endoperoxide PGH_2 (**2**) that occurs readily ($t_{1/2}$ = 5 min at 37 °C) under the aqueous conditions of its cyclooxygenase-promoted biosynthesis from arachidonic acid (AA, **1**).[2] Determining the extent and distribution of levuglandin occurrence *in vivo* is complicated by the fact that LGE_2 binds covalently with proteins.[3] We recently demonstrated that LG-derived protein-bound pyrrole derivatives **4** are major products of this reaction.[4]

LG derivatives may also be formed *in vivo* by nonenzymatic phospholipid peroxidation. Thus, a noncyclooxygenase, free radical pathway generates phospholipid endoperoxides **6** from arachidonyl phospholipids **5** (Scheme 2). The endoperoxides produce racemic 8-epi-PGF_2 isomers *in vivo* that are released upon hydrolysis of plasma phospholipid derivatives **7**.[5] Since PGH_2 rearranges nonenzymatically[2] to LGE_2, an analogous rearrangement of **6** to LG phospholipid derivatives **8** is expected.

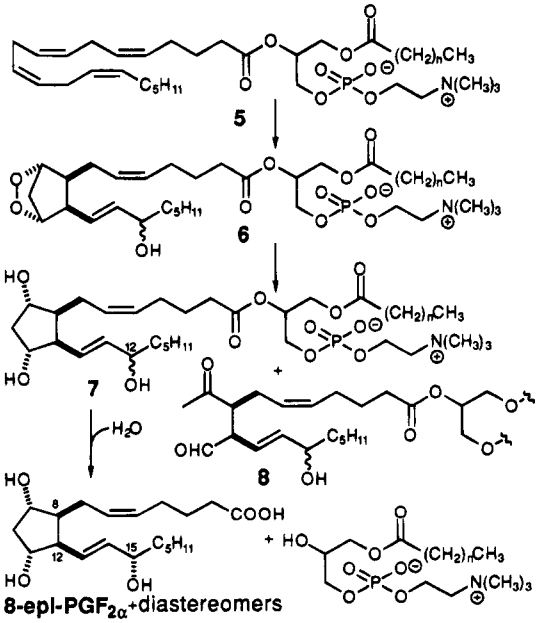
Paal–Knorr condensation[6] of LG phospholipids **8** with protein amino groups would generate pyrrole phospholipids **9** (Scheme 3). Detection of the LG moiety in **9** is especially challenging because it is heavily camouflaged, sandwiched between protein and *lyso*-phosphatidylcholine units. Furthermore, **8** is a mixture of diastereomers that are epimeric with LGE_2 at position 8. However, an important simplification results during conversion to pyrrole **9**. Two of the levuglandin stereocenters in **8** are destroyed. Therefore, hydrolysis of the protein-bound phospholipid **9** will generate the same LG-derived pyrrole **4** as that produced by the cyclooxygenase pathway of Scheme 1.[7]

Previously, we found that protein-bound, LG-derived pyrrole **4** can be detected with the Ehrlich reagent, *p*-(*N*,*N*-dimethylamino)benzaldehyde in the presence of $Et_2O{\cdot}BF_3$. However, the resulting chromophore only allows detection of micromolar concentrations of **4**.[4] An

Scheme 1



Scheme 2



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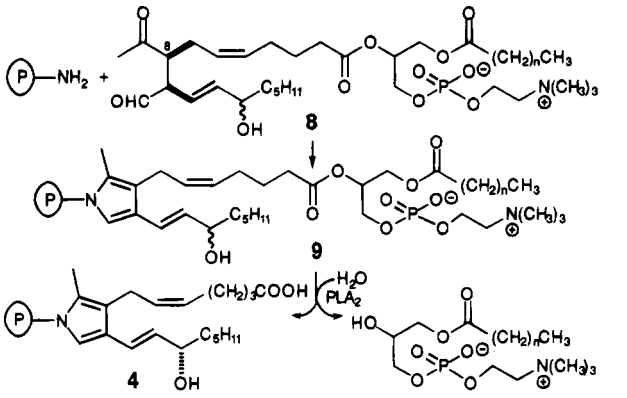
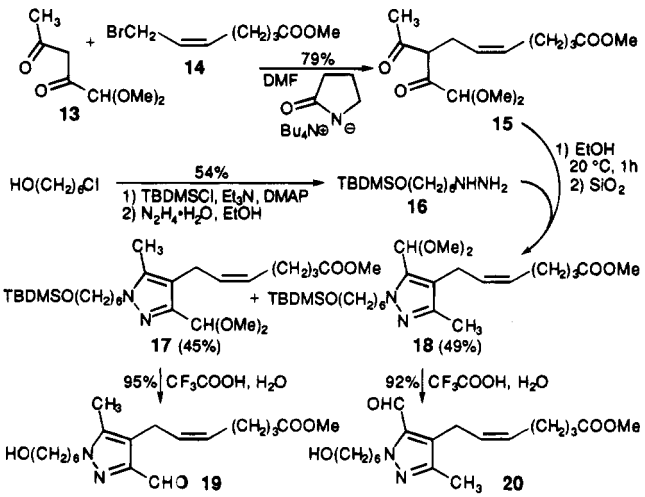
Scheme 3

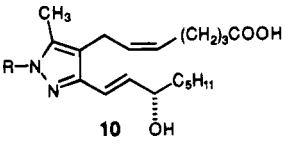


Scheme 4

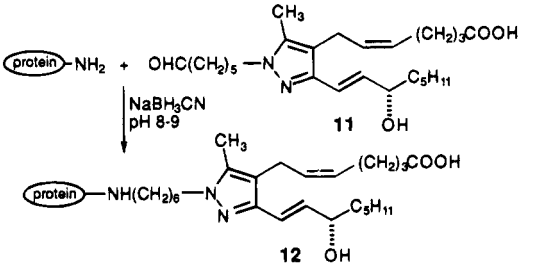


immunoassay with antibodies raised against **4** should provide a more sensitive analytical method for detecting the generation of this pyrrole *in vivo* by the cyclooxygenase pathway. Furthermore, the same immunoassay could be employed to measure the extent and distribution of the *in vivo* occurrence of phospholipid pyrrole **9** after hydrolytic conversion to **4**, e.g., by treatment with phospholipase A_2 (PLA_2).

The limited stability of pyrroles generated by Paal–Knorr condensation of LGE_2 with simple amines[4] discouraged the use of LG-derived protein-bound pyrroles themselves as antigens to raise the requisite antibodies. Instead, we opted for another, more predictably reliable approach that exploits the high crossreactivity anticipated for antibodies raised against a stable isostere of the LG-derived pyrrole. The reactivity of LG-derived pyrroles is dominated by the activating influence of electron donating substituents that can foster electrophilic aromatic substitution or oxidation of the pyrrole ring. In contrast, the isosteric pyrazoles **10** are electron deficient and consequently are resistant to electrophilic aromatic substitution or oxidation. Since pyrroles **4** generated by Paal–Knorr condensation of LGE_2 with the ϵ-amino groups of protein lysyl residues are appended



to the protein by an *n*-alkyl tether to the pyrrole ring nitrogen, the isosteric pyrazole hapten must be linked to a carrier protein by an *n*-alkyl tether to the 1-position of the pyrazole ring. The presence of alkene, carboxyl, and allylic hydroxyl groups limits the choice of reactive functionality that could be exploited to conjugate the hapten with a protein. We now find that an aldehyde group is suitable, allowing coupling by reductive alkylation. Thus, pyrazole isostere hapten **11** was prepared and coupled with poly-L-lysine, bovine serum albumin (BSA), and keyhole limpet haemocyanin (KLH) to provide antigens **12**.



Results and Discussion

Synthesis of a Tetrasubstituted Pyrazole. Condensation of monoalkylhydrazines with 1,3-dicarbonyl compounds provides an expeditious construction of 1-alkylpyrazoles.[8] An appropriate dione (**15**) was assembled by alkylation of 1,1-dimethoxy-2,4-pentanedione (**13**)[9] with bromide **14**[10] (Scheme 4). Outstandingly effective among methods for accomplishing such alkylations[11] was the use of a tetrabutylammonium enolate as nucleophile.[12] Thus, deprotonation of **13** with tetra-*n*-butylammonium 2-pyrrolidonide followed by treatment with allylic bromide **14** in DMF delivered **15**. Although condensation of **15** with monoalkylhydrazine **16** generated a mixture of pyrazoles nonselectively, the desired 5-methylpyrazole **17** was readily separable from its structural isomer **18** by chromatography on silica gel. Hydrolysis of the acetal and silyl ether protecting groups then afforded pyrazole aldehydes **19** and **20**.

Structural Characterization of the Tetrasubstituted Pyrazole Isomers 19 and 20. ^1H NMR correlation spectroscopy (COSY) and nuclear Overhauser enhancement spectroscopy (NOESY)[13] were used to distinguish the pyrazole structural isomers **19** and **20**. COSY correlations facilitated the assignment of ^1H NMR resonances. All correlated coupling partners in the COSY spectra are indicated in Chart 1 with dashed curves. The chemical shifts of corresponding hydrogens in **19** and **20**

(7) The pyrrole **4** from LGE_2 has the *S* configuration at the allylic hydroxyl-bearing carbon while the pyrrole derived from **8** will give both **4** and its *R* epimer upon hydrolysis because **8** and its endoperoxide precursor **6** are expected to be mixtures of racemic diastereomers.

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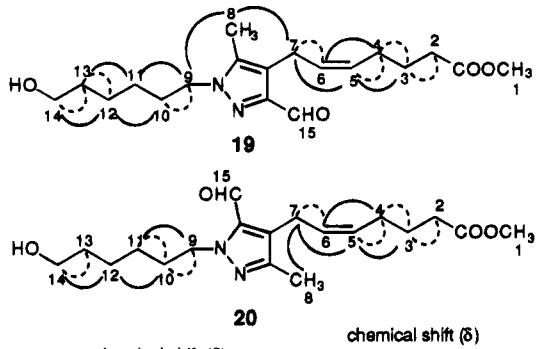
Chart 1. COSY (Dashed Curves) and NOESY (Solid Curves) Correlations and Chemical Shifts of Hydrogens in the ^{1}H NMR Spectra of 19 and 20



H	chemical shift (δ) 19	20	H	chemical shift (δ) 19	20
1	3.68	3.66	8	2.21	2.20
2	2.36	2.34	9	4.09	4.39 ⇦
3	1.72	1.72	10	1.80	1.72
4	2.22	2.19	11	1.40	1.38
5	5.40	5.39	12	1.40	1.38
6	5.40	5.39	13	1.72	1.72
7	3.46	3.38	14	4.34	4.30
			15	9.95	9.85

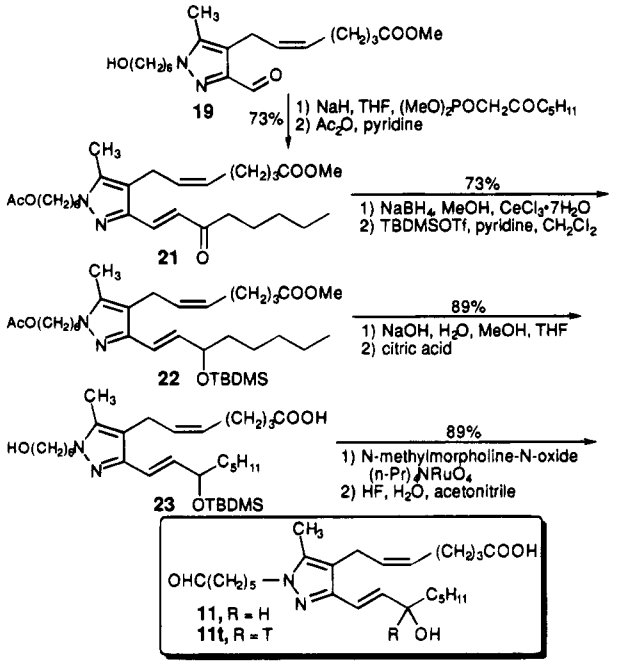
are very similar with the significant exception of the *N*-methylene (H-9) resonance which appears at δ 4.39 for **20** but at δ 4.09 for **19**. The spatial proximity of the *N*-methylene group to the carbonyl group in **20** places the *N*-methylene (H-9) hydrogens in the deshielding cone of the carbonyl group, causing the observed downfield shift of the *N*-methylene signal in **20** relative to **19**.

An especially significant difference in the NOESY spectra of the pyrazole isomers **19** and **20** is the NOE correlation between the methyl hydrogens (H-8) at δ 2.21 and *N*-methylene hydrogens (H-9) at δ 4.09 in **19** and the absence of a correlation between the methyl hydrogens (H-8) at δ 2.20 and *N*-methylene hydrogens (H-9) at δ 4.39 in **20** (Chart 1). This confirms that the methyl group and *N*-alkyl side chain in the isomer assigned structure **20** are further away from each other than the corresponding groups in the isomer assigned structure **19**.

Completion of the Pyrazole Isostere Synthesis. The carbon skeleton was completed by Horner–Emmons coupling of pyrazole aldehyde **19** with dimethyl (2-oxoheptyl)phosphonate (Scheme 5). The primary hydroxyl in the resulting enone was then masked by acetylation to afford **21**. Luche reduction proceeded smoothly to provide a racemic allylic alcohol that was subsequently protected as TBDMS ether **22**. Saponification of both esters in **22** with a 2-fold excess of a 1 M sodium hydroxide solution produced hydroxy acid **23**. Treatment of **23** with common selective oxidants, i.e., pyridinium dichromate, pyridinium chlorochromate, or DMSO–oxalyl chloride–triethylamine, did not provide any of the desired aldehyde **11**.

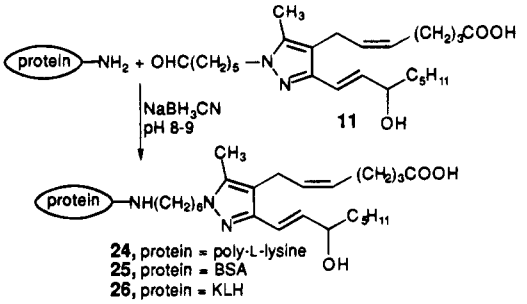
An exceptionally mild method for oxidation of primary alcohols to aldehydes was reported recently.[14] Thus, tetrapropylammonium perruthenate (TPAP) catalyzes a rapid and efficient oxidation of primary alcohols to aldehydes by 4-methylmorpholine *N*-oxide (NMO). Oxidation of **23** was accomplished with TPAP (0.5 mol %), NMO (1.5 equiv), and 4-Å molecular sieves, to remove water generated in the reaction, at room temperature. Treatment of the resulting aldehyde with 15% concentrated hydrofluoric acid in acetonitrile[15] removed the silyl

Scheme 5



ether protecting group to deliver the target pyrazole aldehyde **11**. The synthesis of **11** was readily adapted to the preparation of a tritiated derivative (**11t**) by substituting $NaBT_4$ for $NaBH_4$ in the reduction of **21**.

Conjugation of Isostere Hapten 12 with Proteins. Sodium cyanoborohydride is widely used for imine reduction and reductive amination of proteins in aqueous solution at pH 6–8.[16] Coupling of the aldehyde group in **11** with poly-L-lysine was accomplished in aqueous methanol solution. The heterogeneous reaction mixture became homogeneous as the reaction proceeded. Unreacted starting material, if any, and inorganic salts were removed from the adduct **24** by dialysis (M_r cutoff 14 000) with 90% water in methanol. After removal of solvents, two product fractions were obtained. One is soluble in methanol, the other is insoluble in methanol but soluble in H_2O. The ^{1}H NMR spectrum of the methanol-soluble adduct in CD_3OD clearly shows two absorptions between δ 6.1 and 6.4 corresponding to two vinyl hydrogens in the lower side chain of the pyrazole. Also present is a broad absorption centered at δ 2.85 corresponding to four methylene hydrogens α to the NH group in the adduct **24** and two methylene hydrogens α to unalkylated lysyl NH_2 residues. Since the relative integral areas of the olefinic and methylene absorptions is 1:4, a 1:2 ratio of alkylated versus unalkylated ϵ amino groups or a 1:3 molar ratio of pyrazole to lysyl residues is inferred.



Because NMR spectra of proteins are usually more complex than that of poly-L-lysine, this analytical tech-

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nique is not generally useful for determining the loading of isostere in protein conjugates. Therefore, hapten **11** was tagged with a tiny amount of radiolabeled **11t** and then conjugated with proteins. Thus, BSA was reductively aminated with tagged hapten (specific activity 0.168 mCi/mmol) and sodium cyanoborohydride in water/methanol (4:1). The resulting conjugate **25** was purified by extensive dialysis in phosphate buffer until no unbound radioactivity was present. The molar ratio of BSA:**25** was found to be 1:6.6 by quantitative radiochemical analysis.

Keyhole limpet hemocyanin (KLH) is a protein whose conjugates generally evoke high immunogenic responses.[17] To obtain high incorporation of hapten by reductive alkylation with aldehyde **11**, it was important to use solutions of KLH that had been solubilized in phosphate-buffered saline (PBS).[18] Since organic solvents tended to cause precipitation of KLH, the reductive alkylation was run in PBS with the minimum amount of MeOH required to dissolve the pyrazole aldehyde **11**. Reaction of solubilized KLH and aldehyde **11** with $NaCNBH_3$ followed by dialysis in pH 7.4 PBS gave adduct **26** containing a 0.75:1.0 ratio of hapten to lysyl residues as determined by quantitative radiochemical analysis. The protein–isostere conjugates **24** and **26** both evoke an immune response in rabbits. The preparation of antibodies against these antigens and their use in an enzyme-linked immunosorbent assay that detects protein-bound LG-derived pyrroles will be reported elsewhere.

Experimental Section

General. All proton nuclear magnetic resonance (NMR) spectra were recorded at 200.06 MHz on a Varian XL-200 spectrometer. Proton chemical shifts are reported in parts per million on the δ scale relative to tetramethylsilane (δ 0.00). Tetramethylsilane or chloroform (δ 7.24) was used as internal standard. Significant ^1H NMR spectral data are tabulated in order: multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad), number of protons, and coupling constant(s) in hertz. ^{13}C NMR spectra were recorded on a Varian XL-200 spectrometer at 50.31 MHz. ^{13}C NMR are reported in parts per million on the δ scale relative to chloroform-*d* (δ 77.0). High-resolution mass spectra were recorded on a Kratos/AEI MS-30 dual beam, double focusing magnetic sector mass spectrometer interfaced to a DS-50S Nova-3 computer. Unless otherwise stated, samples were run at 70 eV, 4 kV, 3000 resolution at 3 s per decade. Samples were introduced to the ionization chamber by direct probe insertion.

Thin layer chromatography (TLC) was performed on glass plates precoated with silica gel (kieselgel 60 F_{254}, E. Merck, Darmstadt, West Germany), R_f values are quoted for plates of thickness 0.25 mm. Visualization was done by viewing the developed plate under short-wavelength UV light, by heating the plate after spraying with vanillin–sulfuric acid, or by placing the plate in a chamber filled with iodine vapor. Flash column chromatography was performed on 230–400 mesh silica gel supplied by E. Merck.

Materials. Sodium cyanoborohydride was obtained from Aldrich Chemical Co. Poly-L-lysine and pH 7.4 phosphate buffered saline (PBS) were purchased from Sigma Chemical Co. Spectrapor membrane tubing (M_r cutoff 14 000, no. 2) for standard dialysis was obtained from Fisher Scientific Co. All reactions were performed in an inert moisture-free atmosphere under a positive pressure of nitrogen or argon except when working in aqueous media. Purification and handling of all solvents and reagents used in synthetic procedures were conducted under a nitrogen or argon atmosphere except for aqueous solutions. All solvents were reagent grade or purer. Thiophene-free benzene was boiled under reflux over potassium for several hours and distilled. Hexane and heptane were distilled, stirred over concentrated H_2SO_4 for 1 day, washed with water, saturated $NaHCO_3$, and water, dried over anhydrous $CaCl_2$, boiled under reflux over potassium for 1 day, and redistilled. Tetrahydrofuran (THF) was boiled under reflux over potassium benzophenone ketyl and distilled. Diethyl ether was boiled under reflux over $LiAlH_4$ and distilled. Ethyl acetate, hexane, and diethyl ether used for extractions or chromatography were distilled to remove nonvolatile impurities prior to use.

Methyl 8-Acetyl-10,10-dimethoxy-9-oxo-5(*Z*)-decenoate (15). Into a solution of 1,1-dimethoxy-2,4-pentanedione[9] (**13**, 863.9 mg, 5.43 mmol) in *N*,*N*-dimethylformamide (2.5 mL) was added a stock solution of tetra-*n*-butylammonium 2-pyrrolidonide[12] in *N*,*N*-dimethylformamide (12.49 mL, 5.43 mmol). The solution was stirred 15 min at room temperature, and then methyl 7-bromo-5-heptenoate[10] (**14**, 1.0 g, 4.52 mmol) in *N*,*N*-dimethylformamide (2.5 mL) was added and the reaction stirred another 30 min. TLC analysis in ethyl acetate/hexanes (20% v/v) indicated total disappearance of **14**, excess **13** (R_f = 0.27) and a dark brown-staining spot (R_f = 0.14). The reaction mixture was poured into aqueous ammonium chloride (50 mL) and extracted with diethyl ether (4 × 50 mL). The combined ether extracts were dried over magnesium sulfate, filtered, and concentrated by rotary evaporation to produce an orange liquid. This liquid was purified by flash chromatography (70 mm diameter by 200 mm high column) using ethyl acetate/hexanes (20% v/v) as the eluting solvent. An initial 400 mL was eluted and then fractions (30 × 50 mL) were collected. Fractions 10–24 were pooled and concentrated to afford **15** as a slightly yellow liquid that showed a single spot at R_f = 0.14 upon TLC analysis in the same solvent (987.7 mg, 73% yield): ^1H NMR ($CDCl_3$) δ 5.45–5.25 (2 H, m), 4.41 (1 H, s), 4.00 (1 H, t, J = 7.2 Hz), 3.64 (3 H, s), 3.39 (3 H, s), 3.35 (3 H, s), 2.52–2.44 (2 H), 2.28 (2 H, t, J = 7.5 Hz), 2.21 (3 H, s), 2.12–2.01 (2 H), 1.72–1.57 (2 H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 203.12, 179.74, 173.93, 131.59, 126.00, 103.91, 60.80, 54.98, 51.48, 33.37, 30.54, 26.43, 26.03, 24.56; mass spectrum *m/z* (*M* − 31) calcd for $C_{15}H_{24}O_6$ 269.1389, found 269.1391.

1-(*tert*-Butyldimethylsiloxy)-6-chlorohexane. To a stirred solution of 6-chloro-1-hexanol (8.1 g, 60 mmol), triethylamine (9.2 g, 12.7 mL, 90 mmol), and DMAP (750 mg, 0.1 equiv, 6.1 mmol) in methylene chloride (100 mL) was added *tert*-butyldimethylchlorosilane (10.85 g, 1.2 equiv, 72 mmol). The reaction was allowed to proceed overnight under a nitrogen atmosphere. TLC analysis in ethyl acetate indicated complete disappearance of starting alcohol (R_f = 0.43) and the appearance of a new spot (R_f = 0.67). The organic solution was washed with water (2 × 30 mL) and saturated ammonium chloride solution (1 × 25 mL) and dried over $MgSO_4$. Solvents were removed by rotary evaporation and the residue distilled to afford the title compound (12.03 g, 80%) as a colorless liquid: bp 70 °C/0.1 Torr; ^1H NMR (200 MHz, $CDCl_3$) δ 3.58 (2 H, t, J = 6.26 Hz), 3.50 (2 H, t, J = 6.70 Hz), 1.86–1.69 (2 H), 1.60–1.28 (6 H), 0.86 (9 H, s), 0.02 (6 H, s); mass spectrum *m/z* (M^+) calcd for $C_{12}H_{27}ClOSi$ 250.1520, found 250.1396.

6-(*tert*-Butyldimethylsiloxy)hexylhydrazine (16). To a refluxing solution of 85% hydrazine hydrate (18.0 g, 0.36 mol, 7.5 equiv) in ethanol (30 mL) was added dropwise over 3 h a solution of 1-(*tert*-butyldimethylsiloxy)-6-chlorohexane (12.03 g, 0.048 mmol) in ethanol (40 mL). After the addition was completed, the solution was refluxed for another 3 h. The ethanol was then removed by distillation at atmospheric pressure. To the residue was added saturated aqueous KOH solution (50 mL), and the resulting mixture was extracted with ether (5 × 50 mL). The ether extracts were dried over anhydrous potassium carbonate and filtered, and the ether was removed by rotary evaporation. Fractional distillation of the

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residue under reduced pressure afforded the desired hydrazine **16** (7.90 g, 66.5%) as a colorless liquid: bp 98 °C/0.3 Torr; ^{1}H NMR (200 MHz, $CDCl_3$) δ 3.56 (2 H, t, J = 6.40 Hz), 3.12 (3 H, br s, NH), 2.72 (2 H, t, J = 7.03 Hz), 1.59–1.26 (8 H), 0.85 (9 H, s), 0.00 (6 H, s); mass spectrum m/z (M^+) calcd for $C_{12}H_{30}N_2OSi$ 246.2127, found 246.2110.

Tetrasubstituted Pyrazole Isomers 17 and 18. Monoalkylhydrazine **16** (2.2 g, 9 mmol) was slowly added to a magnetically stirred solution of diketone **15** (1.8 g, 6 mmol) in absolute ethanol (2.5 mL). The resulting reaction mixture was stirred at room temperature for 1 h. Solvent was then removed by rotary evaporation, and the residue was purified by flash chromatography eluting with 20% ethyl acetate in hexane to afford the isomeric pyrazoles **17** (1.40 g) and **18** (1.50 g) in 94% total yield. The ratio of **17**:**18** is 1:1.07.

1-(6-(*tert*-Butyldimethylsiloxy)hexyl)-4-(6-carbomethoxy-2(*Z*)-hexenyl)-3-(dimethoxymethyl)-5-methylpyrazole (17). TLC analysis using ethyl acetate–hexanes 1:4 (v/v) showed a single spot at R_f = 0.08 that was visualized with iodine: ^{1}H NMR (200 MHz, $CDCl_3$) δ 5.40 (m, 3 H), 4.06 (t, 2 H, J = 7.4 Hz), 3.67 (s, 3 H), 3.54 (t, 2 H, J = 6.4 Hz), 3.42 (s, 6 H), 3.39 (d, 2 H, J = 5.2 Hz), 2.40 (t, 2 H, J = 7.5 Hz), 2.20 (m, 2 H), 2.20 (s, 3 H), 1.55 (m, 10 H), 0.87 (s, 9 H), 0.02 (s, 6 H).

1-(6-(*tert*-Butyldimethylsiloxy)hexyl)-4-(6-carbomethoxy-2(*Z*)-hexenyl)-5-(dimethoxymethyl)-3-methylpyrazole (18). TLC analysis using ethyl acetate–hexanes 1:4 (v/v) showed a single spot at R_f = 0.23 that was visualized with iodine: ^{1}H NMR (200MHz, $CDCl_3$) δ 5.40 (m, 2 H), 5.38 (s, 1 H), 4.18 (t, 2 H, J = 7.4 Hz), 3.64 (s, 3 H), 3.60 (t, 2 H, J = 6.4 Hz), 3.35 (s, 6H), 3.21 (d, 2 H, J = 5.2 Hz), 2.38 (t, 2 H, J = 7.5 Hz), 2.16 (m, 2 H), 2.11 (s, 3 H), 1.52 (m, 10 H), 0.88 (s, 9 H), 0.02 (s, 6 H).

A determination of the substitution patterns in these isomeric tetrasubstituted pyrazoles was made by correlation with the derivatives **19** and **20**, respectively. Thus, the isomeric pyrazoles **17** and **18** were characterized further by thorough NMR analysis of the products of hydrolytic removal of the acetal and silyl ether protecting groups.

4-(6-Carbomethoxy-2(*Z*)-hexenyl)-1-(6-hydroxyhexyl)-5-methylpyrazole-3-carboxaldehyde (19). Pyrazole acetal **17** (0.5 g, 0.98 mmol) was treated with 90% TFA (11.0 mL) in water and stirred at room temperature for 30 min. TLC analysis showed one major UV-active spot (R_f = 0.25) with 30% ethyl acetate in hexane. TFA and water were removed by rotary evaporation. The remaining organic residue was purified by flash chromatography with 30% ethyl acetate in hexane as eluting solvent to yield **19** (0.326 g, 95% yield). TLC analysis using ethyl acetate–hexanes 3:7 (v/v) showed a single spot at R_f = 0.25 that was visualized with iodine: ^{1}H NMR (200 MHz, $CDCl_3$) δ 9.95 (s, 1 H), 5.40 (m, 2 H), 4.34 (t, 2 H, J = 6.5 Hz), 4.09 (t, 2 H, J = 7.3 Hz), 3.68 (s, 3 H), 3.46 (d, 2 H, J = 5.3 Hz), 2.36 (t, 2 H, J = 7.4 Hz), 2.22 (m, 2 H), 2.21 (s, 3 H), 1.72 (m, 6 H), 1.40 (m, 4 H): ^{13}C NMR (50 MHz, $CDCl_3$) δ 187.04, 173.35, 143.23, 137.10, 128.10, 127.74, 118.42, 67.32, 50.94, 49.21, 32.85, 29.07, 27.31, 26.03, 25.49, 24.57, 24.17, 20.79, 8.66; mass spectrum m/z (M^+) for $C_{19}H_{30}N_2O_4$ calcd 350.2205, found 350.2197. For NOESY and COSY spectra of **19**, see Chart 1.

4-(6-Carbomethoxy-2(*Z*)-hexenyl)-1-(6-hydroxyhexyl)-3-methylpyrazole-5-carboxaldehyde (20). The procedure was the same as used for making **19**. Pyrazole acetal **18** (0.5 g, 0.98 mmol) gave a crude product that was purified by flash chromatography with 30% ethyl acetate in hexane to afford **20** (0.316 mg, 92% yield). TLC analysis using ethyl acetate–hexanes 1:4 (v/v) showed a single spot at R_f = 0.13 that was visualized with iodine: ^{1}H NMR (200 MHz, $CDCl_3$) δ 9.85 (s, 1 H), 5.39 (m, 2 H), 4.39 (t, 2 H, J = 7.3 Hz), 4.30 (t, 2 H, J = 6.5 Hz), 3.66 (s, 3 H), 3.38 (d, 2 H, J = 5.1 Hz), 2.34 (t, 2 H, J = 7.4 Hz), 2.20 (m, 2 H), 2.19 (s, 3 H), 1.72 (m, 6 H), 1.38 (m, 4 H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 178.96, 173.24, 145.74, 134.33, 128.85, 127.41, 125.70, 67.43, 50.93, 42.80, 32.73, 29.70, 27.27, 26.03, 25.27, 24.45, 23.98, 20.38, 10.82; mass spectrum m/z (M^+) for $C_{19}H_{30}N_2O_4$ calcd 350.2205, found 350.2201. For NOESY and COSY spectra of **20**, see Chart 1.

4-(6-Carbomethoxy-2(*Z*)-hexenyl)-1-(6-hydroxyhexyl)-5-methyl-3-(3-oxo-1(*E*)-octenyl)pyrazole. To a stirred suspension of sodium hydride (59.8 mg of a 50% oil dispersion, 1.247 mmol) in tetrahydrofuran (10 mL) was added dimethyl (2-oxoheptyl)phosphonate (285.7 mg, 1.286 mmol) dropwise over 10 min. This was stirred 4 h at room temperature, and then the thick, white mixture was cooled to −5 °C. Pyrazole aldehyde **19** (95 mg, 0.277 mmol) was dissolved in tetrahydrofuran (5 mL) and also cooled to −5 °C. It was added to the reaction mixture dropwise over 30 min and stirred another 30 min at −5 °C and then at room temperature 20 h. TLC analysis in ethyl acetate/hexanes (75% v/v) showed numerous spots, including a UV-active one that stained greenish-brown in vanillin (R_f = 0.35) that was thought to be the desired product. The tetrahydrofuran was removed by rotary evaporation and the residue taken up in water (25 mL). The aqueous mixture was extracted with diethyl ether (4 × 30 mL), and the combined ethereal extracts were washed with water (50 mL), dried over magnesium sulfate, filtered, and concentrated. The remaining yellow oil was separated by flash chromatography (30 mm diameter by 170 mm high column) utilizing ethyl acetate/hexanes (75% v/v) as eluting solvent. The major UV-active product was collected and solvent was removed by rotary evaporation to yield the title compound as a yellow oil (98.7 mg, 82% yield). TLC analysis using ethyl acetate–hexanes 1:3 (v/v) showed a single spot at R_f = 0.35 that was visualized with vanillin: ^{1}H NMR ($CDCl_3$) δ 7.50 (1 H, d, J = 16.2 Hz), 6.81 (1 H, d, J = 16.2 Hz), 5.41–5.33 (2 H, m), 4.04 (2 H, t, J = 7.4 Hz), 3.69 (3 H, s), 3.63 (2 H, t, J = 6.3 Hz), 3.24 (2 H, d, J = 4.9 Hz), 2.60 (2 H, t, J = 7.5 Hz), 2.38 (2 H, t, J = 7.4 Hz), 2.28–2.16 (2 H), 2.19 (3 H, s), 1.89–1.54 (8 H), 1.51–1.26 (8 H), 0.89 (3 H, t, J = 6.4 Hz); ^{13}C NMR (50 MHz, $CDCl_3$) δ 200.39, 173.40, 143.31, 136.11, 132.13, 128.33, 128.11, 124.84, 118.01, 61.92, 50.91, 48.88, 40.49, 32.84, 31.79, 30.92, 29.53, 26.12, 25.62, 24.56, 24.07, 23.52, 21.89, 21.13, 13.34, 8.87; mass spectrum m/z (M^+) calcd for $C_{26}H_{42}N_2O_4$ 446.3144, found 446.3131.

1-(6-Acetoxyhexyl)-4-(6-carbomethoxy-2(*Z*)-hexenyl)-5-methyl-3-(3-oxo-1(*E*)-octenyl)pyrazole (21). A mixture of the above hydroxy enone (96.9 mg, 0.217 mmol) and pyridine (4.1 mL) was stirred at room temperature. Acetic anhydride (1.11 g, 10.86 mmol) was added dropwise and the reaction stirred for 2 h. TLC analysis in ethyl acetate/hexanes (75% v/v) showed no starting material and just one spot (R_f = 0.50) that was UV-active and stained brown in vanillin. Methanol (2.1 mL) was added and the mixture stirred for 15 h. Methanol and pyridine were removed by rotary evaporation and high vacuum. The crude product was purified by flash chromatography (15 mm diameter by 130 mm high column) using ethyl acetate/hexanes (50% v/v) as the eluant. The major UV-active product was collected and concentrated to afford **21** as a yellowish oil that showed a single spot on TLC analysis (94.1 mg, 89% yield): ^{1}H NMR ($CDCl_3$) δ 7.47 (1 H, d, J = 16.2 Hz), 6.78 (1 H, d, J = 16.2 Hz), 5.39–5.31 (2 H, m), 4.02 (2 H, t, J = 6.5 Hz), 4.00 (4 H, t, J = 7.4 Hz), 3.66 (3 H, s), 3.21 (2 H, d, J = 4.9 Hz), 2.57 (2 H, t, J = 7.5 Hz), 2.35 (2 H, t, J = 7.5 Hz), 2.26–2.13 (2 H), 2.15 (3 H, s), 2.01 (3 H, s), 1.82–1.59 (8 H), 1.36–1.23 (8 H), 0.86 (3 H, t, J = 6.5 Hz); ^{13}C NMR (50 MHz, $CDCl_3$) δ 200.92, 173.95, 169.05, 144.00, 136.60, 132.81, 128.87, 128.73, 125.35, 118.55, 64.24, 51.48, 49.50, 41.06, 33.42, 31.50, 30.10, 28.36, 26.67, 26.27, 25.55, 24.65, 24.10, 22.47, 21.71, 20.96, 13.91, 9.42; mass spectrum m/z (M^+) calcd for $C_{28}H_{44}N_2O_5$ 488.3250, found 488.3244.

1-(6-Acetoxyhexyl)-4-(6-carbomethoxy-2(*Z*)-hexenyl)-3-(3-hydroxy-1(*E*)-octenyl)-5-methylpyrazole. Acetoxy enone **21** (93.0 mg, 0.190 mmol) was dissolved in 0.4 M methanolic cerium(III) chloride heptahydrate (70.9 mg, 0.190 mmol in 475 μL of methanol). Sodium borohydride (7.4 mg, 0.190 mmol) was slowly added, and the reaction was stirred at room temperature for 15 min. TLC analysis in ethyl acetate/hexanes (75% v/v) indicated that the starting material had been totally consumed and there were three products, with one of the spots being more intensely UV-active (R_f = 0.37). Water (15 mL) was added to the reaction mixture, which was then extracted with diethyl ether (3 × 20 mL). The combined ether extracts were dried over magnesium sulfate, filtered, and

concentrated by rotary evaporation. TLC in the same solvent system now showed only the major product spot. The allylic alcohol product was a colorless oil that did not require further purification (75.8 mg, 81% yield): ^{1}H NMR ($CDCl_3$) δ 6.50 (1 H, d, J = 16.0 Hz), 6.27 (1 H, dd, J = 16.0, 6.5 Hz), 5.39–5.31 (2 H, m), 4.22 (1 H, q, J = 6.5 Hz), 4.04 (2 H, t, J = 6.7 Hz), 3.99 (2 H, t, J = 7.4 Hz), 3.69 (3 H, s), 3.17 (2 H, d, J = 4.4 Hz), 2.37 (2 H, t, J = 7.4 Hz), 2.22–2.13 (2 H), 2.15 (3 H, s), 2.04 (3 H, s), 1.83–1.55 (8 H), 1.43–1.23 (10 H), 0.89 (3 H, t, J = 6.4 Hz); ^{13}C NMR (50 MHz, $CDCl_3$) δ 174.02, 145.63, 135.79, 133.10, 129.43, 128.27, 120.93, 115.16, 73.26, 64.36, 51.54, 49.09, 37.26, 33.46, 31.80, 30.28, 28.41, 26.67, 26.32, 25.57, 25.15, 24.70, 22.59, 21.72, 20.96, 14.02, 9.47; mass spectrum m/z (M^+) calcd for $C_{28}H_{46}N_2O_5$ 490.3406, found 490.3405.

1-(6-Acetoxyhexyl)-3-(3-(*tert*-butyldimethylsiloxy)-1(*E*)-octenyl)-4-(6-carbomethoxy-2(*Z*)-hexenyl)-5-methylpyrazole (22). The above allylic alcohol (74.6 mg, 0.152 mmol) and pyridine (60.1 mg, 0.759 mmol) were stirred together in methylene chloride (7.5 mL). *tert*-Butyldimethylsilyl triflate (120.5 mg, 0.456 mmol) was added dropwise, and the reaction mixture was stirred for 3 h at room temperature. TLC analysis in ethyl acetate/hexanes (50% v/v) showed the disappearance of the starting alcohol (R_f = 0.13) and formation of a UV-active spot that was black in vanillin (R_f = 0.58). Methylene chloride and excess pyridine were removed under reduced pressure. The remaining crude product and accompanying white solid were separated by flash chromatography (15 mm diameter by 150 mm high column) with ethyl acetate/hexanes (30% v/v) as the solvent. The major UV-active product was collected and the solvent removed by rotary evaporation to provide **22** as a colorless oil. TLC analysis using ethyl acetate–hexanes 3:7 (v/v) showed a single spot at R_f = 0.30 (75.3 mg, 82% yield): ^{1}H NMR ($CDCl_3$) δ 6.39 (1 H, d, J = 16.2 Hz), 6.15 (1 H, dd, J = 16.1, 5.9 Hz), 5.36–5.29 (2 H, m), 4.17 (1 H, q, J = 6.3 Hz), 4.01 (2 H, t, J = 6.6 Hz), 3.94 (2 H, t, J = 7.4 Hz), 3.65 (3 H, s), 3.14 (2 H, d, J = 5.0 Hz), 2.33 (2 H, t, J = 7.5 Hz), 2.19–2.10 (2 H), 2.11 (3 H, s), 2.01 (3 H, s), 1.78–1.48 (8 H), 1.45–1.22 (10 H), 0.89–0.80 (12 H), 0.02 (3 H, s), 0.00 (3 H, s); ^{13}C NMR (50 MHz, $CDCl_3$) δ 173.89, 170.97, 145.98, 135.73, 133.92, 129.55, 128.15, 119.76, 114.98, 73.80, 64.36, 51.48, 49.10, 38.38, 33.48, 31.85, 30.34, 28.41, 26.72, 26.58, 26.38, 26.16, 25.91, 25.62, 24.92, 24.75, 22.59, 21.82, 20.96, 14.03, 9.45, −4.27, −4.79; mass spectrum m/z (M^+) calcd for $C_{34}H_{60}N_2O_5Si$ 604.4271, found 604.4267.

1-(6-Acetoxyhexyl)-3-(3-(*tert*-butyldimethylsiloxy)-3-tritio-1(*E*)-octenyl)-4-(6-carbomethoxy-2(*Z*)-hexenyl)-5-methylpyrazole (22t). The acetoxy pyrazole enone **21** (40 mg, 0.092 mmol) was dissolved in 500 μL of a solution of cerium(III) chloride in methanol (0.4 M). Solid $NaBT_4$ (2 mg, 0.05 mmol, specific activity 490 mCi/mmol) was carefully added to the reaction mixture, and the mixture was stirred for 30 min at room temperature. TLC analysis in 75% ethyl acetate/hexane showed the presence of a new spot (R_f = 0.35) along with starting material (R_f = 0.5). Water (4 mL) was added to the reaction mixture, which was extracted with diethyl ether (5 × 5 mL). The combined ether extracts were dried ($MgSO_4$), filtered, and concentrated in vacuo. The crude residue was purified by silica gel chromatography with 75% ethyl acetate/hexane as eluant to give the desired α-tritio alcohol (14.8 mg, 83% based on consumed starting enone) as a colorless oil and the starting enone **21** (18.9 mg). The specific activity of the product was determined as follows. The product was dissolved in 10 mL of ethyl acetate, and a 50 μL aliquot was withdrawn from it and further diluted to 1 mL (200×) with ethyl acetate. Three 100 μL aliquots of this diluted solution were counted in a liquid scintillation counter. The average count was 724 366 dpm. From this the specific activity of the alcohol was found to be 21.8 mCi/mmol. This α-tritio alcohol was treated with *tert*-butyldimethylsilyl triflate as for the unlabeled alcohol above to deliver the title silyl ether **22t**.

3-(3-(*tert*-Butyldimethylsiloxy)-1(*E*)-octenyl)-4-(6-carboxy-2(*Z*)-hexenyl)-1-(6-hydroxyhexyl)-5-methylpyrazole (23). Pyrazole diester **22** (73.0 mg, 0.121 mmol), tetrahydrofuran (2.7 mL), and methanol (4.0 mL) were stirred while an aqueous 1 M NaOH solution (14.15 mg, 0.363 mmol, 363 μL of solution) was slowly added, and the mixture was then stirred at room temperature for 4 h. TLC analysis in ethyl acetate/hexanes (75% v/v) showed a small amount of starting acetoxy ester (R_f = 0.81), a presumed intermediate (R_f = 0.49), and possibly the desired product as a streak (R_f = 0.05 to 0.18). (Note: acids are known to streak on TLC plates in certain solvents.) An additional quantity of 1 M NaOH (9.7 mg, 0.242 mmol, 242 μL of solution) was added and the reaction stirred another 2 h. TLC analysis in 89% hexanes/10% 2-propanol/1% acetic acid (v/v) showed mainly one product (R_f = 0.14). The reaction mixture was acidified to pH 5 with a saturated aqueous citric acid solution and extracted with ethyl acetate (3 × 20 mL). The combined organic extracts were washed with water (2 × 20 mL), dried over magnesium sulfate, and filtered, and the solvent was removed by rotary evaporation. The crude product was purified by flash chromatography (15 mm diameter by 140 mm high column) with a solvent of 89% hexanes/10% 2-propanol/1% acetic acid (v/v/v). The major UV-active product was collected, and solvents were removed to provide **23** as a pale yellow oil that showed a single spot on TLC analysis (58.9 mg, 89% yield): ^{1}H NMR ($CDCl_3$) δ 6.40 (1 H, d, J = 16.1 Hz), 6.15 (1 H, dd, J = 16.0, 5.9 Hz), 5.38–5.31 (2 H, m), 4.19 (1 H, q, J = 5.6 Hz), 3.97 (2 H, t, J = 7.3 Hz), 3.59 (2 H, t, J = 6.3 Hz), 3.15 (2 H, d, J = 4.4 Hz), 2.34 (2 H, t, J = 7.5 Hz), 2.20–2.10 (2 H), 2.11 (3 H, s), 1.78–1.67 (4 H), 1.55–1.46 (4 H), 1.39–1.18 (10 H), 0.89–0.81 (12 H), 0.04 (3 H, s), 0.02 (3 H, s); ^{13}C NMR (50 MHz, $CDCl_3$) δ 177.74, 145.86, 135.90, 134.15, 129.49, 128.33, 119.65, 115.10, 73.86, 62.43, 48.86, 38.33, 33.30, 32.26, 31.85, 30.28, 26.61, 26.08, 25.92, 25.02, 24.50, 22.64, 21.83, 18.28, 14.08, 9.49, −4.26, -4.80; mass spectrum m/z (M^+) calcd for $C_{31}H_{56}N_2O_4Si$ 548.4009, found 548.3967.

3-(3-(*tert*-Butyldimethylsiloxy)-1(*E*)-octenyl)-4-(6-carboxy-2(*Z*)-hexenyl)-5-methyl-1-(6-oxohexyl)pyrazole. The alcohol **23** (20 mg, 0.036 mmol) was dissolved in dichloromethane (4 mL) containing 4-Å molecular sieves and 4-methylmorpholine *N*-oxide (6.3 mg, 0.054 mmol). Solid tetrapropylammonium perruthenate (2 mg, 0.15 equiv) was then added under nitrogen and the resulting green mixture stirred at room temperature. After 1 h of stirring, TLC analysis showed a new spot (R_f = 0.3) with 70% ethyl acetate in hexane. Evaporation and filtration (small pipette silica gel column) eluting with ethyl acetate removed all the inorganic material. Rotary evaporation gave a crude siloxy aldehyde (14 mg, 70% yield) as an oil. This crude product was used for the next reaction without further purification: ^{1}H NMR (200 MHz, $CDCl_3$) δ 9.73 (t, 1 H, J = 1.6 Hz), 6.40 (d, 1 H, J = 16.0 Hz), 6.16 (dd, 1 H, J = 16.08, 5.96 Hz), 5.34 (m, 2 H), 4.20 (m, 1 H), 3.96 (t, 2 H, J = 7.45 Hz), 3.15 (d, 2 H, J = 4.69 Hz), 2.39 (m, 4 H), 2.20 (m, 2 H), 2.11 (s, 3 H), 1.50 (m, 16H), 0.88 (s, 9 H), 0.85 (t, 3 H, J = 2.24 Hz), 0.042 (s, 3 H), 0.02 (s, 3 H).

4-(6-Carboxy-2(*Z*)-hexenyl)-3-(3-hydroxy-1(*E*)-octenyl)-5-methyl-1-(6-oxohexyl)pyrazole (11). The above TBDMS ether (14 mg, 0.025 mmol) was treated with concentrated aqueous hydrofluoric acid (0.16 mL, 49% w/v) and acetonitrile (0.34 mL) in a polyethylene vial. The desilylation was followed by TLC with ethyl acetate as developing solvent (R_f of starting silyl ether 0.57, desilyated product 0.24). After 20 min, TLC analysis showed no starting material. The reaction mixture was diluted with water (1.5 mL), extracted with $CHCl_3$ (3 × 5 mL), and dried over anhydrous $MgSO_4$. Filtration and evaporation gave hydroxy aldehyde **11** as a slightly yellow oil. This could be purified by silica gel chromatography (pipette column) using ethyl acetate as the mobile phase to give **11** as a single spot by TLC analysis using ethyl acetate as developing solvent R_f = 0.25 (9.6 mg, 87% yield): ^{1}H NMR (200 MHz, $CDCl_3$) δ 9.76 (t, 1 H, J = 1.6 Hz), 6.48 (d, 1 H, J = 16.2 Hz), 6.19 (dd, 1 H, J = 16.2, 5.99 Hz), 5.36 (m, 2 H), 4.22 (m, 1 H), 3.99 (t, 2 H, J = 7.46 Hz), 3.16 (d, 2 H, J = 4.71 Hz), 2.40 (m, 4 H), 2.24 (m, 2 H), 2.12 (s, 3 H), 1.50 (m, 16 H), 0.86 (t, 3 H, J = 2.25 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 202.45, 177.14, 145.60, 136.02, 133.09, 129.34, 128.57, 120.70, 115.49, 73.30, 52.67, 49.07, 48.82, 43.65, 37.19, 33.17, 31.79, 30.36, 26.46, 25.18, 24.44, 22.62, 21.69, 14.08, 9.57; mass spectrum m/z (M^+) for $C_{25}H_{40}N_2O_4$ calcd 432.2988, found 432.2899.

Reductive Alkylation of Poly-L-lysine with Aldehyde 11. Poly-L-lysine (2.7 mg, 4 equiv based on unit base of lysine, M_r = 55 000) and pyrazole aldehyde **11** (2 mg, 0.0046 mmol) were dissolved in methanol (0.4 mL). This solution became a little cloudy. After 5 min of stirring, sodium cyanoborohydride (1.0 mg) was quickly added at room temperature. When the addition was complete, the solution became clear. This solution was stirred for 2 h at room temperature. After 2 h, TLC analysis with ethyl acetate showed a new UV-active polar spot and the disappearence of **11** (R_f = 0.24). The solution was transferred to a dialysis tube (M_r cutoff 14 000, Spectrapor membrane tubing no. 2) and dialyzed twice against 10% water (250 mL) in methanol for 24 h. The absence of free hapten **11** in the polylysine conjugate **24** was confirmed by TLC with ethyl acetate as developing solvent. After dialysis and concentration of adduct by rotary evaporation, two product fractions were obtained. One (2.3 mg) is soluble in MeOH, the other (2.5 mg) is insoluble in MeOH but soluble in water.

Reductive Alkylation of Bovine Serum Albumin with Pyrazole Aldehyde 11. BSA (5.5 mg, 0.085 mmol) and **11** containing a small amount of the allylically tritiated derivative **11t** (4.4 mg, 10.2 mmol, specific activity 0.168 mCi/mmol, prepared from a mixture of **22** and **22t**) were dissolved in a solution of water (1.6 mL) and methanol (450 μL), and the reaction mixture was stirred for 10 min at room temperature. Solid sodium cyanoborohydride (5 mg, 86 mmol) was added to the reaction mixture and stirring was continued for 6 h. The reaction mixture was then transferred to a dialysis tube and dialyzed against 500 mL of pH 7.4 PBS buffer for 36 h, changing the buffer every 12 h. After dialysis, a TLC analysis showed no starting aldehyde or sodium cyanoborohydride. The solvent was removed in vacuo and the residue, BSA adduct **25**, was dissolved in PBS pH 7.4 (2.5 mL). Two 50 μL aliquots were counted (average dpm = 4150) and the molar ratio of BSA:pyrazole (hapten) was calculated to be at least 1:6.6.

Reductive Alkylation of KLH with Pyrazole Aldehyde 11. A mixture of ^{3}H-labeled **11t** and unlabeled aldehyde **11** (4.4 mg, 0.010 mmol, specific activity = 0.092 mCi/mmol) in MeOH (400 μL) was added to KLH (10.7 mg, 700 μL of a solution containing 15.3 mg KLH/mL PBS).[18] The solution was stirred for 10 min, and then $NaBH_3CN$ was added (5 mg, 0.080 mmol) and the reaction mixture was then stirred for 16 h. (**Note:** a minimum of organic solvent must be used to avoid the precipitation of the KLH from solution.) The entire reaction mixture was then placed in dialysis tubing (M_r cutoff = 12 000−14 000) and stirred in PBS (3 × 400 mL) for 48 h, replacing the PBS solution after 8 and 24 h. The dialyzed suspension was diluted to a total volume of 3 mL, and two aliquots (30 μL) were counted; 40% (0.0040 mmol) of the starting aldehyde **11** had been incorporated into the protein conjugate **26** as determined by a count of 3.72×10^{-4} mCi for the entire mixture.

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Supplementary Material Available: ^{1}H NMR spectra of all new compounds (16 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.