

**SYNTHESIS OF ^{13}C SINGLE AND DOUBLE LABELLED RETINALS,
PRECURSORS FOR NMR STUDIES OF VISUAL PIGMENTS AND RELATED SYSTEMS[†]**

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SUMMARY

The synthesis of retinals enriched with ^{13}C at positions 11, 13, 14, 15, 12&20, 13&14, and 14&15 in high isomeric purity is reported. The importance of these retinals in studying the structure and properties of rhodopsin and bacteriorhodopsin by ^{13}C NMR spectroscopy is briefly described.

Key Words: ^{13}C enriched retinals, Emmons-Horner reaction, Arbusov reaction, Manganese(IV) oxide oxidation, Allylic bromination.

INTRODUCTION AND DISCUSSION

^{13}C NMR is a powerful tool for investigations of the structure and properties of visual pigments and related systems (rhodopsin and bacteriorhodopsin).^(1,2) ^{13}C chemical shifts and coupling constants reflect local electronic and steric effects and, as such, are sensitive to the inductive, steric, and coulombic interactions which may play important roles in the wavelength regulation in visual transduction.⁽³⁾ Moreover, ^{13}C nuclear magnetic resonance (NMR) presents the advantage of probing exclusively the ground state of these molecules, thus avoiding possible formation of photointermediates. The synthetic enrichment of retinals with ^{13}C , their use in regenerating the rhodopsin and bacteriorhodopsin pigments from the corresponding apoproteins, and the low natural abundance (1.1%) of ^{13}C makes it possible to observe in the NMR spectrum of the pigment the signals of the specifically labeled positions.^(4,5) Moreover, it has been recently shown that application of modern NMR techniques such as double-quantum coherence spectroscopy with double labelled retinals leads to useful simplification of ^{13}C spectra of biomacromolecules.⁽⁶⁾

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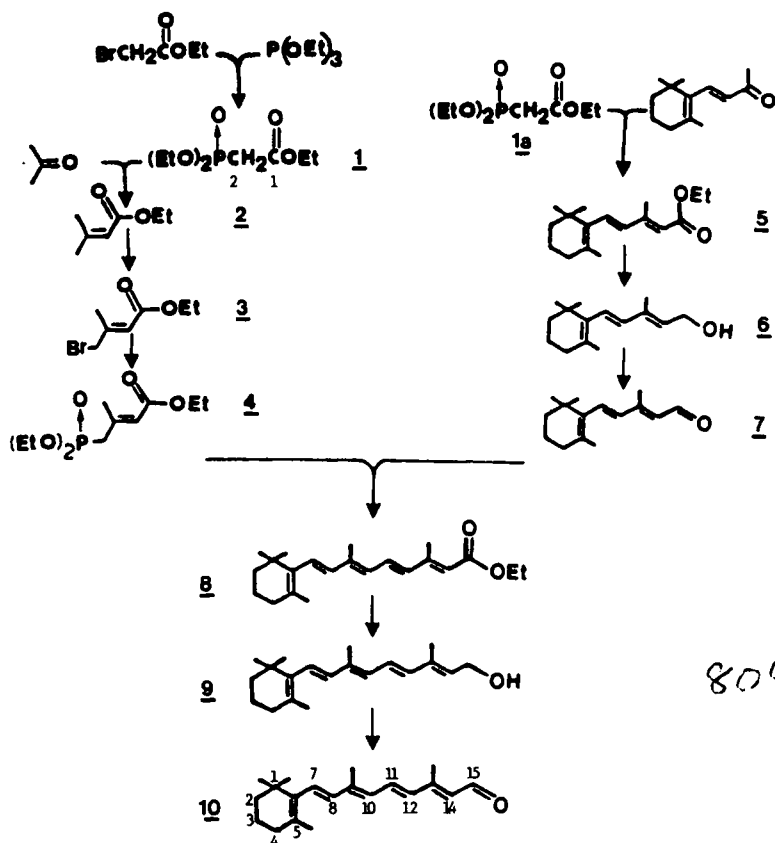
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To carry out these NMR investigations, it was necessary to design an efficient, reliable synthetic procedure to obtain high yield, high purity ^{13}C enriched retinal isomers. We report here the synthesis of retinals enriched in positions 11,13,14,15, 12&20, 13&14, and 14&15 according to a β -ionylidene acetaldehyde isoprenylation route (Scheme 1). This scheme, based on the Emmons-Horner reaction,⁽⁷⁾ is characterized by high versatility. Positions 10 through 15 may be enriched in very good yields by choosing the appropriate labelled precursors. The reactions are stereoselective, as the all-trans isomer predominates in the final product, despite the fact that the ethyl- γ -diethyl phosphonosencioate **4** is a cis-trans (40:60) mixture.⁽⁸⁾

Although a number of synthetic routes are reported in literature for the synthesis of retinals and their derivatives,⁽⁹⁾ the unique feature of this synthetic scheme is its adaptation for specific ^{13}C labelling with consideration for the availability and cost of ^{13}C enriched precursors. By choosing the appropriate ^{13}C precursors, β -C15 aldehyde **7** can also be enriched in positions 10 and 11. The efficiency and versatility of this synthetic scheme for ^{13}C labelled retinals have made practical ^{13}C NMR studies of visual pigments and related systems.^(1,4)

EXPERIMENTAL

Ethyl bromoacetate (90% ^{13}C enriched at positions 1 or 2, and 99% at 1&2, MSD Isotopes) was used without further purification. Unlabelled ethyl bromoacetate (Aldrich) was distilled in vacuo (bp=62-3°C, 19mm Hg) before use. Triethyl phosphite (Aldrich) was distilled at atmospheric pressure (bp=150°C). Tetrahydrofuran (THF) and diethyl ether (Et_2O) were freshly distilled prior to use under a nitrogen atmosphere from sodium metal and lithium aluminum hydride (LiAlH_4), respectively. Hexamethylphosphoric triamide (HMPA, Aldrich) was distilled from barium oxide (BaO) under nitrogen. Acetone (90% ^{13}C enriched at positions 2 or 1&3, MSD Isotopes) was used without further purification. Acetone (spectrophotometric grade, Aldrich) was distilled under nitrogen atmosphere and stored over 4Å molecular sieves. β - Ionone (Aldrich) was distilled in vacuo (bp=126-128°C, 12 mm Hg) and stored in brown bottles under an atmosphere of



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Scheme 1. Synthesis of ¹³C enriched retinals. Labeled precursors and the corresponding enriched positions are listed below, with ¹³C chemical shifts of the label in all-trans-retinals.

Precursor	Enriched Retinal Position	Chemical Shift
A. 1- ¹³ C-ethylbromoacetate, 1	15	191.4
B. 2- ¹³ C-ethylbromoacetate, 1	14	129.0
C. 1,2- ¹³ C ₂ -ethylbromoacetate, 1	14,15	*
D. 1- ¹³ C-ethylbromoacetate, 1a	11	132.5
E. 2- ¹³ C-acetone	13	154.1
F. B and E	13,14	*
G. 1,3- ¹³ C ₂ -acetone	12,20	*

*See Figure 3

nitrogen. Hexane was distilled over sodium hydride before use. Manganese(IV) oxide (activated, Aldrich) was used as such for the oxidation reactions. Lithium aluminum hydride (LiAlH_4), 1.0M solution in diethyl ether was an Aldrich product. All reagents were stored and handled under dry nitrogen atmosphere. All glassware was oven dried at 120°C overnight before use. Thin layer chromatography (TLC) plates (silica gel 60 F-254, 0.25 mm, Merck) were used for monitoring reactions. Silica gel (Merck 230-400 Å) was used for purification of reaction mixtures by flash column chromatography.⁽¹⁰⁾

Products were characterized by ^1H and ^{13}C NMR using Varian XL-200 and Bruker WH270 spectrometers. All the spectra were taken in CDCl_3 with TMS as an internal reference. The ^{13}C and ^1H NMR spectra of labelled retinals and retinal precursors were compared with reference spectra of unlabelled material.⁽¹¹⁾ These data were used to confirm structural assignments and the level of isotopic enrichment. UV-Visible spectra were obtained in spectrophotometric cyclohexane or ethanol solutions using a Cary 15 spectrophotometer. Absorption spectra of synthetic intermediates and labelled retinals were identical with those reported in literature.⁽¹²⁾

Triethyl phosphonoacetate 1 via Arbusov Reaction⁽¹³⁾

A three neck flask equipped with a magnetic stirrer, reflux condenser, and pressure-equalizing addition funnel, was charged under nitrogen atmosphere with triethyl phosphite (41.5g, 42.9 ml, 0.25 mole) and heated to 60°C with stirring. Ethyl bromoacetate (36.76g, 25 ml, 0.22 mole) was added dropwise to the stirring triethyl phosphite over a period of 30 min. and heated to 80°C for three hours. The reaction mixture was cooled to room temperature, and the traces of ethyl bromide and excess triethylphosphite were removed in vacuo (80°C , 0.5mm Hg). Triethyl phosphonoacetate was obtained in 99.5% yield (50.19g, 0.224 mole, see Figure 1).

^{13}C enriched triethyl phosphonoacetate was synthesized by condensing triethyl phosphite with the appropriate ^{13}C enriched ethylbromoacetate under similar conditions as used for the preparation of unlabelled triethyl phosphonoacetate. Proton NMR spectra of the enriched compounds are shown in Figure 1, where

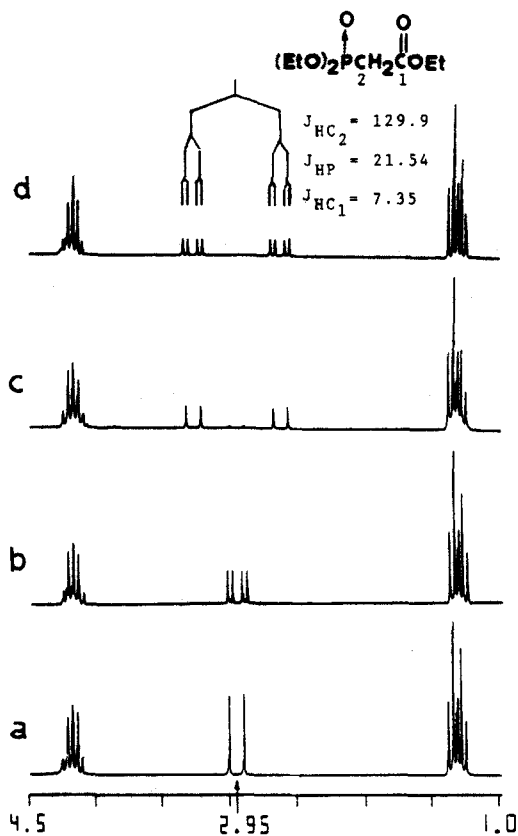


Figure 1. 200 MHz proton NMR spectra of: (a) triethylphosphonoacetate, (b) [1- ^{13}C]triethylphosphonoacetate, (c) [2- ^{13}C]triethylphosphonoacetate, and (d) [1,2- $^{13}\text{C}_2$]triethylphosphonoacetate. Coupling constants are in Hz.

the splittings due to ^{13}C - ^1H coupling are evident. The level of isotopic enrichment was determined by integration of corresponding signals. The isotopic purity of the enriched precursors was maintained during this and subsequent synthetic steps.

Ethyl seneciatoate 2 via the Horner ylide

Sodium hydride (50% in oil, 285.6mg, 5.95 mmole) was washed with hexane and THF under nitrogen atmosphere and suspended in 5 ml THF. Triethyl phos-

phonoacetate (1.33g, 5.95 mmole) was added dropwise at 0°C and stirred (2 hours) until the solution was clear and pale yellow. To this solution, acetone (345.5mg, 5.95 mmole, 436.9 ul) was added dropwise via syringe at 0°C and the reaction mixture stirred for 2-3 hours after warming to room temperature. The reaction mixture was cooled to 0°C and the excess hydride was quenched with water (5ml). The product was extracted with Et₂O (3x15ml), washed with water (3x10ml), and the combined organic layers were dried over anhydrous sodium sulfate. The solvent was removed in mild vacuum to yield ethyl senecioate (735mg, 5.73 mmole, 96%).

¹³C enriched ethyl senecioates were prepared under similar conditions starting from ¹³C triethyl phosphonoacetate or ¹³C acetone.

¹H NMR: (δ 1.2t, 3H), (1.8s, 3H), (2.17s, 3H), (4.15q, 2H), (5.68s, 1H).

¹³C NMR: 166.77, 156.34, 116.66, 59.60, 27.52, 20.23, 14.66 ppm.

Ethyl-γ-bromosenecioate 3 via allylic bromination⁽¹⁴⁾

To a solution of ethyl senecioate (735 mg, 5.73 mmole) in carbon tetrachloride (CCl₄, 5ml) was added N-bromosuccinimide (1.03g, 5.79 mmole) and a few crystals of Azobis(isopropyl cyanide) as a catalyst. The reaction mixture was boiled under reflux at 90°C for 2-3 hours, at which time succinimide had formed a layer at the top of pale yellow solution. The reaction mixture was cooled to room temperature, filtered through a sintered glass funnel and concentrated in vacuo to yield cis and trans ethyl-γ-bromosenecioate (1.156g, 5.56 mmole, 97%).

¹H NMR: (δ 1.3t, 3H), (2.0s, 3H), (2.2s, 3H), (3.9s, 2H), (4.1q, 2H), (4.5s, 2H), (5.9d, 1H).

Ethyl-γ-diethyl phosphonoseneioate 4

Triethyl phosphite (970mg, 5.83 mmole, 1.00 ml) was heated to 80°C with stirring and ethyl-γ-bromosenecioate (1.156g, 5.56 mmole) was added dropwise via syringe. The reaction mixture was refluxed at 90°C for 3 hours. After this time it was cooled to room temperature, and the traces of ethyl bromide and excess triethylphosphite were removed in vacuo (80°C, 0.4mm Hg) to yield cis and trans (40:60) ethyl-γ-diethylphosphonoseneioate (1.44g, 5.44 mmole, 98%).

Ethyl- β -ionylideneacetate 5

Sodium hydride (50% in oil, 6.92g, 0.144 mole) was suspended in THF (20ml) after washing with hexane and THF. Triethyl phosphonoacetate (40.5g, 35.85ml, 0.181 mole) was added dropwise over a period of sixty minutes at 0°C. The reaction mixture was stirred until the formation of a clear light yellow solution. To the yellow anionic solution was added HMPA (10ml), the flask shielded from light with aluminum foil and treated dropwise with β -ionone (25.0g, 26.56 ml, 0.13 mole) at 0°C. The reaction mixture was slowly allowed to attain room temperature and stirred for 24 hours. The excess hydride was quenched with water (90ml) at 0°C and the product extracted with ether (3x100ml). The combined organic layer was washed with water, dried over sodium sulfate and concentrated in vacuo to yield ethyl- β -ionylideneacetate (33.06g, 0.126 mole, 97%), λ_{max} = 258 and 304 nm in ethanol.

Purification, if necessary, was done by flash column chromatography using 8% Et_2O in hexane as eluent.

 β -ionylidene ethanol 6

To a solution of β -ionylidene acetate (26.54g, 0.101 mole) in ether (100 ml) at -70°C was added dropwise a solution (1M) of lithium aluminum hydride in ether (25.25 ml, 0.101 mole). The reaction mixture was warmed to -25°C and stirred for thirty minutes at this temperature. The reaction was monitored by TLC using 50% ether in hexane as developing solvent (R_f β -ionylidene ethanol=0.32, R_f β -ionylideneacetate=0.61). A slight excess of lithium aluminum hydride may be used when required to drive the reaction to completion. At the time the reaction was complete, the reaction mixture was cooled to -70°C and the excess hydride was quenched by dropwise addition of methanol (20ml) followed by addition of water (100ml). The aqueous mixture was extracted with ether (3x100ml), the organic extract combined and dried over sodium sulfate and concentrated in vacuo to obtain the β -ionylidene ethanol (22.30g, 0.101mole, 100%). This compound was used immediately for oxidation without purification. λ_{max} 235 and 255 in hexane.

 β -ionylidene acetaldehyde 7

To a solution of β -ionylidene ethanol (21.5g, 97.7 mmole) in dichlo-

romethane (200ml) was added manganese(IV) oxide (66.0g, 0.75 mole, 8-fold excess). The reaction mixture was stirred for 8 hours and monitored by TLC using 50% ether in hexane, the product forming a yellow spot, after spraying with H_2SO_4 . Upon completion, the mixture was filtered through a celite cake and washed thoroughly with dichloromethane and once with ether. The solution was concentrated in vacuo and the residue purified by flash column chromatography using 5% ether in hexane as eluent to yield β -ionylidene acetaldehyde (21.30g, 93.0 mmole, 93% yield). The product is predominantly the all-trans isomer (See Figure 2).

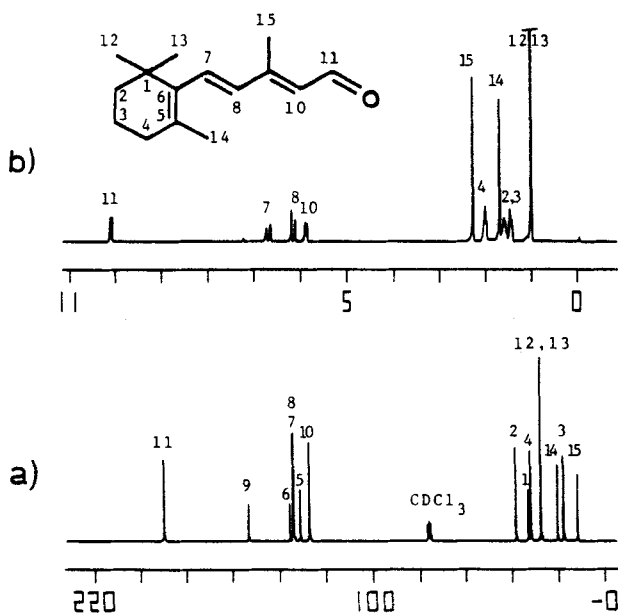


Figure 2. (a) The 50.3 MHz ^{13}C NMR spectra and (b) the 200 MHz proton spectra of β -ionylidene acetaldehyde.

Ethyl retinoate 8

Sodium hydride (50% in oil, 144.0mg, 3.02 mmole) was suspended in THF (5ml) after washing with hexane and THF, and 1.12ml HMPA was added to this suspension. Ethyl- γ -diethyl phosphoseneoate (794.4 mg, 699 ml, 3 mmole) was added drop-

wise at 0°C. Hydrogen evolution was observed with the deepening of the color of the solution to amber. The reaction mixture was stirred at 0°C until clear. The flask was shielded from light with aluminum foil and treated dropwise with β-ionylidene acetaldehyde (654.0mg, 733.5 ul, 3 mmole) using THF (2ml) to carry and wash. The reaction mixture was allowed to react at 0°C for 24 hours. The workup was carried out as for 5 with ether/water, the combined organic layers were dried over Na₂SO₄ and concentrated in vacuo to yield ethylretinoate (1.063g crude) with a trace of β-C₁₅ aldehyde at R_f=0.50 (yellow/H₂SO₄).

Purification was carried out by flash column chromatography using 1.5% Et₂O/hexane as mobile phase. Purified product (534mg, 57%), λ_{max}=345nm (hexane), R_f=0.71 (red spot/H₂SO₄).

Retinol 9

To a solution of ethyl retinoate 8 (534mg, 1.12 mmoles) in ether (10ml) at -70°C under nitrogen was added dropwise a solution of lithium aluminum hydride (1M) in ether (1.4 ml, 1.12 mmoles) in dark. The reaction mixture was stirred and allowed to warm to -25°C. The reaction was monitored by TLC, with the formation of a product indicating a blue green spot at R_f=0.17 when sprayed with H₂SO₄. A slight excess of LiAlH₄ may be used if required, for the completion of the reaction. The workup was done as for 6 and the product dried over Na₂SO₄ under N₂ atmosphere, yield=385 mg, 1.01 mmole, 90%, λ_{max}=326nm (hexane) indicative of clean product. This compound was used immediately without further purification.

Retinal 10

Retinol, 9, (385mg, 1.0 mmole) was taken up in 5 ml dichloromethane, in a flask shielded with aluminum foil and manganese(IV) oxide (2g, 8.3 mmole) was added in portions to the solution at 0°C. The reaction mixture was stirred for 7-8 hours and monitored by TLC, the product forming yellow spots at R_f= 0.35 and 0.41 (mixture of two isomers) after spraying with H₂SO₄. At the completion of the reaction, it was filtered in dim light and washed well with dichloromethane. The solution was concentrated in vacuo; the crude product (364mg, 0.93 mmoles, 92%) was predominantly all-trans (λ_{max} = 368 nm in cyclohexane). Purification

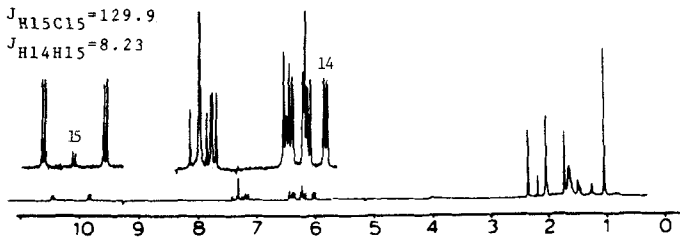


Figure 3. The 270 MHz ^1H spectrum of all-trans-[15- ^{13}C]retinal with inserts showing expansion of spectral regions revealing ^{13}C - ^1H coupling.

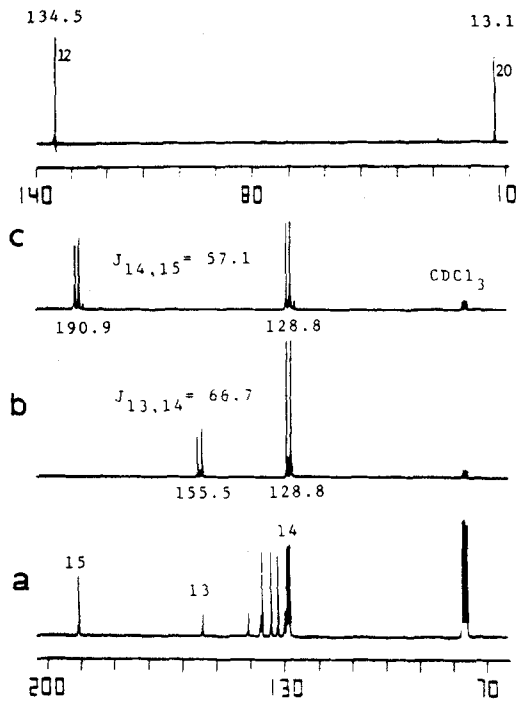


Figure 4. Low field region of the 50.3 MHz ^{13}C NMR spectra of (a) all-trans-retinal, (b) all-trans-[13,14- $^{13}\text{C}_2$]retinal, and (c) all-trans-[14,15- $^{13}\text{C}_2$]retinal. Also included is the spectrum of [12,20- $^{13}\text{C}_2$]retinal obtained as described in the text.

and separation of isomers was carried out by HPLC using hexane:ether:THF (89%:10%:1%) on a 50 cm Magnum 20 silica column (Whatman Partisil 10). Representative ¹H and ¹³C NMR spectra and relevant chemical shift and coupling values of enriched retinals are given in Figures 3 and 4.

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REFERENCES

1. Shriver, J.W., Fager, R., Torchia, D., Abrahamson, E.W. and Mateescu, G.D., *Nature*, **270**, 271 (1977)
2. Shriver, J.W., Mateescu, G.D. and Abrahamson, E.W., *Meth. Enzym.*, **81**, 698 (1982)
3. Shriver, J.W., Mateescu, G.D. and Abrahamson, E.W., *Biochemistry*, **18**, 4785 (1979)
4. Mateescu, G.D., Abrahamson, E.W., Shriver, J.W., Copan, W.G., Muccio, D.D., Iqbal, M., and Waterhous, V., in "Spectroscopy of Biological Molecules", C. Sandorfy and Th. Theophanides, Eds., Reidel: Dordrecht, Boston, pp 250-283 (1984)
5. Mateescu, G.D., Copan, W.G., Muccio, D.D., Waterhous, V.D., and Abrahamson, E.W., in "Synthesis and Applications of Isotopically Labelled Compounds", W. Duncan & A. Susan, Eds., Elsevier: Amsterdam, New York, pp. 123-132 (1983)
6. Mateescu, G.D., Iqbal, M., and Wheeler, C., *Biophys. J.*, in press
7. a) Pattenden, G. and Weedon, B.C.L., *J. Chem. Soc. (C)*:1984 (1968)
b) Derguini, F., Balogh-Nair, Valeria, and Nakanishi, K., *Tetrahedron Lett.*, 4899 (1979)
c) Kaegi, H., Chew, E., and Chien, P.L., *J. Labelled Compds. Radiopharm.*, **17** 745 (1980)
8. Gedye, R. N., Arora, P., and Khalil, A.H., *Can. J. Chem.* 1943 (1975)
9. a) Kaegi, H. H. and DeGraw, J. L., *J. Labelled Compds. Radiopharm.* **18**, 1099 (1980)
b) Bu'lock, J. D., Quarrie, S. A. and Taylor, D. A., *ibid*: **9** 311 (1973)
c) Ronnie, L. H., Walter, B., Clark, W. P. and Arnold A. L., *ibid.*, **13**, 123 (1976)
d) Von Den Tempel, P. J. and Huisman, H. O., *Tetrahedron* **22**, 293 (1966)
e) Broek, A. D. and Lugtenburg, J., *Recl. Trav. Chim. Pays-Bas*, **99**, 363 (1980); *ibid* **101**, 102 (1982)
10. Clark Still, W.C., Khan, M., and Mitra, A., *J. Org. Chem.* **43**, 2923 (1978)
11. a) Shriver, J.W., Abrahamson, E.W., and Mateescu, G.D., *J. Am. Chem. Soc.*, **98**, 2407 (1976)
b) Copan, W.G., Ph.D. Dissertation, Case Western Reserve University, 1982
12. Schwieter, U. and Isler, O., in "The Vitamins", V.I, Sebrell, Ed., Academic Press, New York (1967)
13. a) Pattenden, G. and Weedon, B.C.L., *J. Chem. Soc. (C)*: 1997 (1968)
b) Burrell, J.W.K., Garwood, R.F., Jackman, L.M., Oskay, E., and Weedon, B.C.L., *J. Chem. Soc. (C)*: 2144 (1966)
14. a) Ziegler, K., Spath, A., Schaaf, E., Schuman, W., and Winkelmann, E., *Annalen*, **557**, 80 (1942)
b) Stotter, P.L. and Hill, K.A., *Tetrahedron Lett.* 1679 (1975)