Journal of Chromatography, 625 (1992) 163–168
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 414

Effect of temperature on chiral and achiral separations of diacylglycerol derivatives by high-performance liquid chromatography on a chiral stationary phase

Toru Takagi and Toshiyuki Suzuki

Department of Chemistry, Faculty of Fisheries, Hokkaido University, Hakodate 041 (Japan)

(First received April 8th, 1992; revised manuscript received June 3rd, 1992)

ABSTRACT

Enantiomer separation of 1,2-diacyl-*rac*-glycerols (1,2-*rac*-DGs) as their 3,5-dinitrophenylurethane derivatives by high-performance liquid chromatography was carried out on a Sumichiral OA-4100 column. The separation of 1,2-DG enantiomers was improved by lowering the temperature. Linear relationships were found between the logarithm of the separation factor and the reciprocal of absolute temperature. Using thermodynamic concepts, detailed considerations are presented for the enantiomer separation and separation of molecular species differing in carbon number or olefinic bond number. Separation by carbon number is controlled by entropy differences, whereas separation by double bond number is based on enthalpy contributions.

INTRODUCTION

Recently, we reported the direct enantiomer separation of 1,2-diacylglycerols (1,2-DGs) as their 3,5-dinitrophenylurethane (3,5-DNPU) derivatives by high-performance liquid chromatography (HPLC) on the chiral stationary phases (CSPs) Sumichiral OA-2100 and OA-4100 [1–3]. The structures of the 1,2-DG 3,5-DNPU derivatives and OA-4100 stationary phase are shown in Fig. 1. The separation of enantiomers by HPLC using CSPs is based on the formation of transient diastereomeric complexes between the enantiomorphs of the solute and a chiral selector of the CSP. Typically defined chemical structures, such as π-acidic or π-basic aromatic groups and polar hydrogen bond donors/acceptors, near the chiral center, participate in the multiple attractive interactions.

All HPLC separations of 1,2-DG 3,5-DNPUs reported previously were carried out at ambient temperature. In this study, enantiomer separation was significantly improved by operating at low temperature. The selectivity, α, is related to temperature (T) and free energy difference ($\Delta\Delta G°$) between analytes: $\ln \alpha = -\Delta\Delta G°/RT$. Variations of the equation give the enthalpy ($\Delta\Delta H°$) and entropy ($\Delta\Delta S°$) differences. These values are obtained by plotting

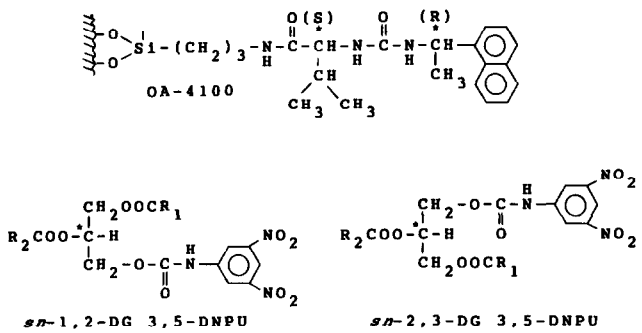

Fig. 1. Structures of Sumichiral OA-4100 stationary phase and enantiomers of 1,2-DG 3,5-DNPU derivatives.

Correspondence to: Dr. Toru Takagi, Department of Chemistry, Faculty of Fisheries, Hokkaido University, Hakodate 041, Japan.

ln α *vs.* $1/T$. This approach has been applied to understanding chiral recognition [4,5].

In addition to chiral separations, the CSP used in this study provides achiral separations based on the number of carbon atoms and the total number of double bonds in the two constituent fatty acids. In spite of the intensive efforts that have been devoted to the study of chiral recognition on CSPs, achiral recogniton on CSPs has hardly been considered. We now present detailed thermodynamic considerations of DG enantiomer separations and achiral separations of DG molecular species differing in the number of acyl carbons and olefinic bonds.

EXPERIMENTAL

Samples

Enantiomers of 1,2- and 2,3-*sn*-DGs were synthesized by the method of Howe and Malkin [6]. DG racemates were obtained by inter-esterification of fatty acid methyl esters with glycerol in dimethylformamide medium at 90°C. The separation of 1,2-*rac*-DGs was carried out by thin-layer chromatography (TLC) on silica gel G impregnated with boric acid, using hexane–diethyl ether (60:40, v/v) and chloroform–acetone (96:4, v/v) as developing solvents. Conversion of 1,2-*rac*-DGs into 3,5-DNPU derivatives was carried out as described in previous papers [7,8].

HPLC

HPLC separation was carried out with a Hitachi (Tokyo, Japan) L-6200 instrument equipped with a Sumichiral OA-4100 chiral column (stainless steel, 50 cm × 4 mm I.D.) using hexane–1,2-dichloroethane–ethanol as the eluent at a constant flow-rate. Peaks were monitored with a Shimadzu (Kyoto, Japan) SPD-6A UV detector at 254 nm. A Hitachi Model 638-0805 recycle valve was used for recycling. The column temperature was maintained by dipping in an ethanol bath, which was cooled by an immersion cooler (Tokyo Rika, Tokyo, Japan) to within about 1.0°C with an exclusive controller.

Definitions

The structures of the fatty acids considered are expressed as follows: 16:0 = $CH_3(CH_2)_{14}COOH$; 18:0 = $CH_3(CH_2)_{16}COOH$; 20:0 = $CH_3(CH_2)_{18}COOH$; 22:0 = $CH_3(CH_2)_{20}COOH$; 18:1 = $CH_3(CH_2)_7CH = CH(CH_2)_7COOH$; 18:2 = $CH_3(CH_2)_4CH = CHCH_2CH = CH(CH_2)_7COOH$; and 18:3 = $CH_3CH_2CH = CHCH_2CH = CHCH_2CH = CH(CH_2)_7COOH$.

RESULTS AND DISCUSSION

Fig. 2 shows the HPLC separation of a saturated 1,2-*rac*-DG homologue mixture containing four acyl groups (16:0–18:0–20:0–22:0) as their 3,5-DNPU derivatives on Sumichiral OA-4100. In a previous study, we reported the separation of ten saturated enantiomers containing three acyl groups (16:0–18:0–20:0) [3]. In this study, complete separation of fourteen enantiomers was achieved at low temperature (Fig. 2B). These fourteen enamtiomer

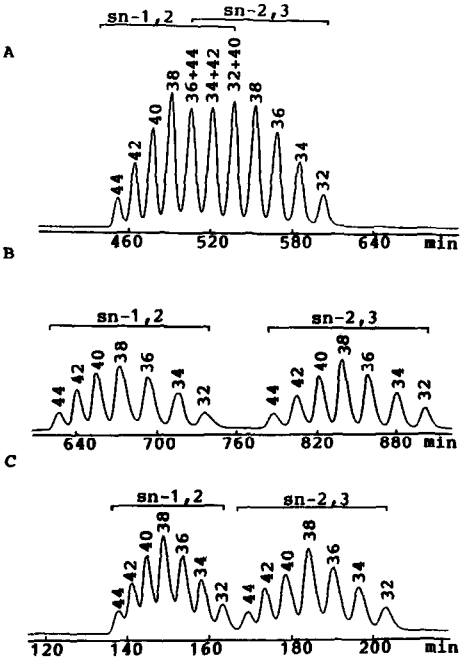

Fig. 2. Separation of saturated DG enantiomers as 3,5-DNPU derivatives on an OA-4100 chiral column. Temperature: (A) 19.5; (B) −23.0; (C) −20.0°C. Mobile phase: hexane–1.2-dichloroethane–ethanol, (A) and (B) 170:10:1 (v/v/v) and (C) 150:20:1 (v/v/v). Flow-rate: (A) and (B) 0.25 and (C) 0.5 ml/min. Peaks were monitored at 254 nm after the first recycle. Above each peak is given the acyl carbon number of the DG. 32 = C_{16} + C_{16}; 34 = C_{16} + C_{18}, C_{18} + C_{16}; 36 = C_{18} + C_{18}, C_{16} + C_{20}, C_{20} + C_{16}; 38 = C_{16} + C_{22}, C_{22} + C_{16}, C_{18} + C_{20}, C_{20} + C_{18}; 40 = C_{18} + C_{22}, C_{22} + C_{18}, C_{20} + C_{20}; 42 = C_{20} + C_{22}, C_{22} + C_{20}; 44 = C_{22} + C_{22}.

peaks were not separated at ambient temperature (Fig. 2A).

The separation factors (α) and peak resolutions (R_s) between 1,2- and 2,3-enantiomers increased from 1.13–1.14 to 1.24–1.28 and from 4.33–5.07 to 9.64–11.13, respectively, on lowering the temperature (Fig. 2A and B). On the other hand, the values of α and R_s for homologues differing by two acyl carbons were independent of temperature (Fig. 2A, α = 1.03, R_s 1.12–1.38; Fig. 1B, α = 1.02–1.03, R_s = 1.01–1.38). To obtain a rapid separation, the HPLC condition was changed (Fig. 2C). All the racemic DGs were separated into fourteen enantiomers within 220 min at −20°C.

Fig. 3 shows separation of 1,2-*rac*-DGs prepared from 18:0 and 18:1 acids. Better enantiomer resolution was obtained at low temperature. In contrast to the separation of homologues differing by two carbons, the α and R_s values obtained in the separation of DG molecular species differing by one double bond increased slightly at low temperature (Fig. 3A, α = 1.03–1.04, R_s = 0.95–1.19; Fig. 3B, α = 1.04–1.05, R_s = 1.11–1.45).

Fig. 4 shows a plot of ln α *vs.* $1/T$ for 1,2-di-18:0-*rac*-DG enantiomers. A similar linear relationship

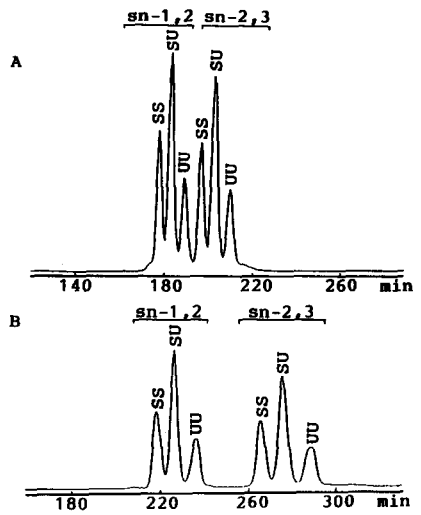

Fig. 3. Separation of DG enantiomers prepared from 18:0 and 18:1 acids as 3,5-DNPU derivatives on an OA-4100 chiral column. Temperature: (A) 27.0 and (B) −10.5°C. Mobile phase: hexane–1,2-dichloroethane–ethanol (170:10:1, v/v/v) at a flow-rate of 0.25 ml/min. SS = di-18:0-DG; SU = 18:0–18:1-DG; UU = di-18:1-DG. Peaks were monitored at 254 nm.

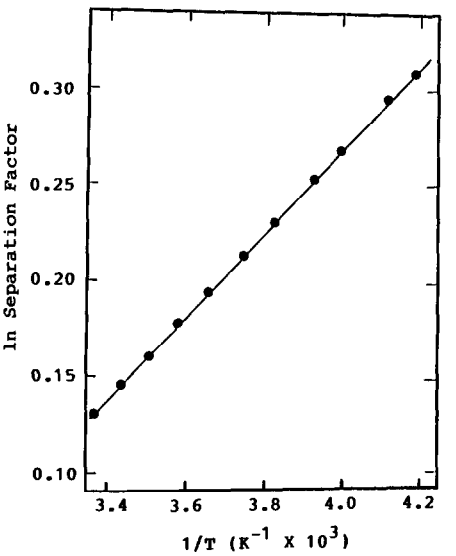

Fig. 4. Plot of ln α *vs.* $1/T$ for 1,2-di-18:0-*rac*-DG enantiomers.

was also obtained for enantiomers of various DG molecular species. This linear relationship is explained by the following well known thermodynamic equation [9]:

$$\ln \alpha = -\Delta\Delta G^\circ/RT \quad (1)$$

where $\Delta\Delta G^\circ$ is the free energy difference between the analytes and R is gas constant. Eqn. 1 and also be expressed application of the Gibbs–Helmholtz equation, $G = H - TS$, as follows:

$$\ln \alpha = -\Delta\Delta H^\circ/RT + \Delta\Delta S^\circ/R \quad (2)$$

Thus, the differences in enthalpy ($\Delta\Delta H^\circ$) and entropy ($\Delta\Delta S^\circ$) between the analytes were calculated from the slope and intercept of the line with the vertical axis.

Thermodynamic data obtained for enantiomers of various DG molecular species are given in Table I. Although the CSP gives almost the same enantioselectivity for these DGs at 25°C, the values of $\Delta\Delta H^\circ$ and $\Delta\Delta S^\circ$ increased slightly with increase in degree of unsaturation. Introduction of a *cis* double bond gives various conformations to the acyl chain [10], and the acyl conformation may produce slight differences in $\Delta\Delta H^\circ$ and $\Delta\Delta S^\circ$ on enantiomer separation.

The CSP used in this study has a polar functional

TABLE I

ENTHALPY AND ENTROPY DIFFERENCES OBTAINED IN ENANTIOMER SEPARATIONS

Mobile phase: hexane–1,2-dichloroethane–ethanol (170:10:1, v/v/v) at a flow-rate of 0.25 ml/min.

Molecular species *sn*-2,3/*sn*-1,2	α (25°)	$\Delta\Delta G°$ (25°C) (cal/mol)	$\Delta\Delta H°$ (cal/mol)	$\Delta\Delta S°$ (cal/mol · K · 0.1)
18:0–18:0	1.13	−70.39	−396.98	−10.96
18:0–18:1	1.13	−70.43	−414.92	−11.56
18:0–18:2	1.12	−68.24	−418.69	−11.76
18:0–18:3	1.12	−68.00	−428.53	−12.10
18:1–18:1	1.13	−71.56	−407.36	−11.27
18:2–18:2	1.13	−69.86	−430.17	−12.09
18:3–18:3	1.12	−68.92	−428.53	−12.07

group, N-(*R*)-1-(α-naphthyl)ethylaminocarbonyl-(*S*)-valine chemically bonded to silanized silica. This functional group provides separations according to both carbon number and degree of unsaturation. DG molecular species elute from the CSP in order of decreasing carbon number and increasing number of double bonds (Figs. 2 and 3). In this study, the temperature dependences of α on carbon number and double bond number differences were also determined. The effect of temperature on these achiral separations was extremely slight. Hence changing the temperature is not a practical means of improving the achiral separations, but out of interest we observed the temperature dependence of α.

Table II shows the thermodynamic parameters obtained in the achiral separations. The values of $\Delta\Delta H°$ based on carbon number difference were much smaller than those based on double bond difference. This indicates that separation according to carbon number is nearly independent of temperature, whereas that according to the number of double bonds is dependent on temperature.

The values of $\Delta\Delta G°$ and $\Delta\Delta H°$ were plotted against the differences in carbon number and degree of unsaturation (Figs. 5 and 6, respectively). Al-

TABLE II

ENTHALPY AND ENTROPY DIFFERENCES OBTAINED IN SEPARATIONS BASED ON CARBON NUMBER AND DOUBLE BOND NUMBER

Mobile phase: hexane–1,2-dichloroethane–ethanol (170:10:1, v/v/v) at a flow-rate of 0.25 ml/min.

Molecular species *sn*-2,3	α (25°C)	$\Delta\Delta G°$ (25°C) (cal/mol)	$\Delta\Delta H°$ (cal/mol)	$\Delta\Delta S°$ (cal/mol · K · 0.01)
20:0–18:0/20:0–20:0	1.03	−18.23	9.55	9.32
18:0–18:0/20:0–20:0	1.06	−36.44	2.98	13.23
18:0–16:0/20:0–20:0	1.10	−56.44	5.39	20.75
16:0–16:0/20:0–20:0	1.14	−78.20	5.52	28.09
18:0–18:1/18:0–18:0	1.03	−19.96	−34.87	−5.01
18:0–18:2/18:0–18:0	1.09	−52.13	−64.22	−4.06
18:1–18:1/18:0–18:0	1.08	−43.11	−73.57	−10.22
18:0–18:3/18:0–18:0	1.15	−82.60	−116.91	−11.52
18:2–18:2/18:0–18:0	1.20	−110.23	−155.88	−15.32
18:3–18:3/18:0–18:0	1.34	−173.85	−251.25	−25.97

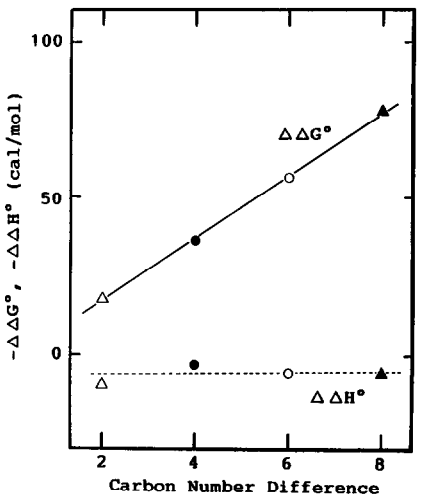

Fig. 5. Plots of $\Delta\Delta G^\circ$ and $\Delta\Delta H^\circ$ *vs.* difference in acyl carbon number between DG molecular species. △ = 20:0–18:0-DG/di-20:0-DG; ● = di-18:0-DG/di-20:0-DG; ○ = 18:0–16:0-DG/di-20:0-DG; ▲ = di-16;0-DG/di-20:0-DG.

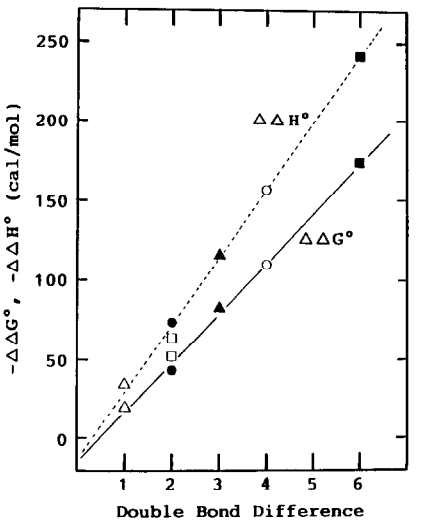

Fig. 6. Plots of $\Delta\Delta G^\circ$ and $\Delta\Delta H^\circ$ *vs.* difference in number of double bonds between DG molecular species. △ = 18:0–18:1-DG/di-18:0-DG; ● = di-18:1-DG/di-18:0-DG; □ = 18:0–18:2-DG/di-18:0-DG; ▲ = 18:0–18:3-DG/di-18:0-DG; ○ = di-18:2-DG/di-18:0-DG; ■ = di-18:3-DG/di-18:0-DG.

though $\Delta\Delta G^\circ$ increased with increase in carbon number difference, the $\Delta\Delta H^\circ$ values were nearly constant (Fig. 5). This indicates that the contribution of $\Delta\Delta H^\circ$ to the separation according to the carbon number is relatively unimportant, and the separation is controlled by $\Delta\Delta S^\circ$ based on shape selectivity. In contrast, separation according to the number of double bonds is controlled by $\Delta\Delta H^\circ$ (Fig. 6), and here the contribution of $\Delta\Delta S^\circ$ is cancelled by the large value of $\Delta\Delta H^\circ$. The increase in $\Delta\Delta H^\circ$ with increase in the difference in double bond number suggests a binding interaction between the CSP and double bonds, and the binding interaction is responsible for the separation according to degree of unsaturation on this CSP.

TABLE III

ENTHALPY AND ENTROPY DIFFERENCES OBTAINED IN SEPARATIONS BASED ON CARBON NUMBER AND DOUBLE BOND NUMBER

Mobile phase: hexane–1,2-dichlorethane–ethanol (170:10:1, v/v/v) at a flow-rate of 0.25 ml/min.

Molecular species *sn*-2,3	α (25°C)	$\Delta\Delta G^\circ$ (25°C) (cal/mol)	$\Delta\Delta H^\circ$ (cal/mol)	$\Delta\Delta S^\circ$ (cal/mol · K · 0.01)
20:0–18:0/20:0–20:0	1.03	−17.06	−3.75	4.47
18:0–18:0/20:0–20:0	1.06	−35.78	−2.82	11.06
18:0–16:0/20:0–20:0	1.10	−55.36	−6.84	16.28
16:0–16:0/20:0–20:0	1.14	−75.98	−10.33	22.03
18:0–18:1/18:0–18:0	1.03	−19.92	−16.60	1.11
18:0–18:2/18:0–18:0	1.09	−51.99	−45.95	2.03
18:1–18:1/18:0–18:0	1.07	−41.95	−63.19	−7.13
18:0–18:3/18:0–18:0	1.15	−83.18	−98.85	−5.26
18:2–18:2/18:0–18:0	1.20	−108.48	−126.13	−5.92
18:3–18:3/18:0–18:0	1.34	−173.66	−235.59	−20.78

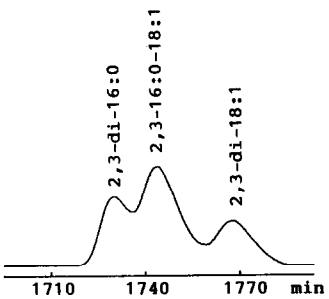

Fig. 7. Separation of critical pair prepared from 16:0 and 18:1 acids as 3,5-DNPU derivatives on an OA-4100 chiral column. Mobile phase: hexane–1,2-dichloroethane–ethanol (170:5:1, v/v/v) at a flow-rate of 0.25 ml/min. Peaks were monitored at 254 nm after the third recycle.

Dipole–induced dipole type hydrogen bonds are formed between polar X–H groups and polarizable multiple bonds such as isolated double bonds and benzene rings. The CSP used in this study contains NH groups on a chiral selector and OH groups on a silica support. A binding interaction may occur between the positively charged hydrogen of the X–H groups and π electron clouds on the double bonds. While this characteristic difference in separation according to carbon number and degree of unsaturation was also observed for the *sn*-1,2-enantiomer group (Table III), there is a problem with the results of these achiral separations. Tables II and III present the same results, but concerning the corresponding antipodes; the selectivity values are identical and also the $\Delta\Delta G^\circ$ values because enantiomers exhibit the same physico-chemical properties. Nevertheless, the data show differences in the enthalpy and entropy values for the two antipodes. The reason why the $\Delta\Delta H^\circ$ and $\Delta\Delta S^\circ$ values observed for two enantiomers are so different is unknown.

In a previous study, critical pairs were found with the DGs such as the pair of 1,2-16:0–18:0-DGs and 1,2-18:1–18:0-DGs corresponding to the same equivalent carbon number (*ECN*) [3]. These critical pairs were not separated at ambient temperature. The *ECN* value was calculated from the total acyl carbon number (*CN*) and the total number of double bonds (n) of the two constituent fatty acids according to the equation

$$ECN = CN - 2n \quad (3)$$

In this study, critical pairs were separated at low temperature. Fig. 7 shows the separation of the critical pair of *sn*-2,3-di-16:0, 2,3-16:0–18:1- and 2,3-di-18:1-DGs. The column temperature was maintained at -12.5°C for recycle chromatography. The peak area ratio is 1:2:1, and the second peak is assumed to be a mixture of *sn*-2-16:0–3-18:1- and *sn*-2-18:1–3-16:0-DGs, but detailed identification was not carried out.

In conclusion we have described the effect of temperature on DG enantiomer separation. The resolution was much improved at low temperature, but the column temperatures below -30°C caused abnormal pressure increases, irregularity of the chromatographic curve and/or deformation of peaks, hence temperatures below -30°C cannot be used for the usual analyses on this CSP. Another aspect considered is the essential difference in the mechanisms of separations according to carbon number and number of double bonds. Separation according to carbon number is controlled by $\Delta\Delta S^\circ$, whereas separation according to degree of unsaturation is based on the contribution of $\Delta\Delta H^\circ$. This characteristic difference may be expected for the other chromatographic separations.

REFERENCES

1 Y. Itabashi and T. Takagi, *J. Chromatogr.*, 402 (1987) 257.
2 T. Takagi and Y. Itabashi, *Lipids*, 22 (1987) 596.
3 T. Takagi and T. Suzuki, *J. Chromatogr.*, 519 (1990) 237.
4 W. H. Pirkle and T. C. Pochapsky, *J. Chromatogr.*, 369 (1986) 175.
5 W. H. Pirkle and T. C. Pochapsky, *Chem. Rev.*, 89 347 (1989).
6 R. J. Howe and T. Malkin, *J. Chem. Soc.*, (1951) 2663.
7 N. Oi and H. Kitahara, *J. Chromatogr.*, 265 (1983) 117.
8 N. Oi and H. Kitahara, *J. Liq. Chromatogr.*, 9 (1986) 511.
9 S. G. Allenmark, *Chromatographic Enantioseparation: Methods and Applications*, Ellis Horwood, Chichester 1988, pp. 70–71.
10 R. R. Brenner, *Prog. Lipid Res.*, 23 (1984) 69.