Structures of fucose-containing ceramide pentasaccharides from the plasma of blood group O Le(a⁻b⁻) nonsecretors

Gerhard Pfannschmidt, Jasna Peter-Katalinić*, Maria Kordowicz, Heinz Egge*, Janusz Dabrowski+, Ursula Dabrowski* and Peter Hanfland†

*Institut für Experimentelle Hämatologie und Bluttransfusionswesen der Universität Bonn, *Institut für Physiologische Chemie der Universität Bonn, Nussallee 11, 5300 Bonn 1 and +Max-Planck-Institut für Medizinische Forschung, Jahnstraße 29, 6900 Heidelberg, FRG*

A minor, Le^c blood-group inactive ceramide pentasaccharide double band fraction has been isolated from the plasma of blood group O Le(a⁻b⁻) nonsecretors. The two purified glycolipids were analysed by NMR spectroscopy, mass spectrometry and combined gas chromatography–mass spectrometry. The following structures could be established: GlcNAc(β1→3)Gal(β1→4)Glc(β1→1)Cer (I); Gal(β1→4)[Fuc(α1→3)]GlcNAc(β1→3)Gal(β1→4)Glc(β1→1)Cer (II). It must be concluded that at least part of the secretor gene-independent plasmatic H type 2 blood-group activity can be attributed to glycosphingolipid I, whereas substance II, originally detected in cancerous tissue, also occurs in the plasma of healthy individuals.

Blood group   Glycosphingolipid   Human plasma   Lewis negative (non)secretor

1. INTRODUCTION

The Le^a, Le^b and Le^d (H type 1) blood-group active glycosphingolipids of human erythrocytes and plasma have been identified in [1–5]. The Le^c property has hitherto been defined only by agglutination of Le(a⁻b⁻) erythrocytes of adult nonsecretors with appropriate antisera of human [6] or goat [7] origin. It has been suggested that the Le^c antigens are represented by either lacto-N-fucopentaosyl (III)- [6,8], or unsubstituted lacto-N-tetraosyl-oligosaccharides [9]. Immunochemical studies, however, excluded this correlation, since corresponding glycolipids failed to react with the above-mentioned antisera [10]. During the course of our studies on the isolation and characterization of Le^c active glycosphingolipids from the plasma of Le(a⁻b⁻) nonsecretors (in preparation) we identified a fraction containing two fucolipids with a chromatographical migration behaviour similar to that of Le^a or Le^d (H type 1) active glycosphingolipids. This paper describes the isolation and complete immuno- and physicochemical characterization of these two fucolipids by means of haemagglutination inhibition tests, ^{1}H NMR, fast atom bombardment (FAB) and electron impact (EI) mass spectrometry and combined gas chromatography–mass spectrometry (GC–MS). The resulting structures allow conclusions to be drawn with regard to the interrelationships of the ABH-, Lewis and secretor/nonsecretor blood-group systems.

† To whom correspondence should be addressed

Abbreviations: Abbreviations and trivial names are essentially the same as in [3]: Gal(β1→4)[Fuc(α1→3)] GlcNAc(β1→3)Gal(β1→4)Glc, lacto-N-fucopentaose III [19]; Fuc(α1→2)[Gal(β1→4)]GlcNAc(β1→3)Gal(β1→4)Glc, lacto-N-fucopentaose IV; amu, atomic mass units

2. EXPERIMENTAL

2.1. Materials

Glycosphingolipids as reference substances and an *Ulex europaeus* lectin preparation were essentially the same as in [3]. Human anti-Le^c ('ARM') [6] was a generous gift from Dr R.J. Crawford (Glasgow and West of Scotland Blood Transfusion Service, Carluke, Scotland) and goat anti-Le^c [7] was obtained from Dr H.A. Graham (Ortho Diagnostic Systems Inc., Raritan, NJ).

2.2. Isolation and purification of glycosphingolipids

Plasma (15 l) collected from 5 blood-group O Le(a$^-$b$^-$) nonsecretor individuals by repeated plasmapheresis was lyophilized and stored until use. The isolation procedure was described previously [1,3]. Purification was completed by preparative silica gel HPTLC [1] of the peracetylated glycolipids on 20 × 20 plates using dichloromethane–methanol–water (97:3:0.2, v/v) as solvent system followed by deacetylation [11].

2.3. Biological assays

Serological activities against anti-Le^c sera and *U. europaeus* lectin were tested by the haemagglutination inhibition test [1,2,12]. Le^c reactivity was also checked by the passive haemagglutination technique as in [1,2,12].

2.4. Methylation analysis

Permethylation of glycosphingolipids, subsequent derivatization to partially methylated neutral and amino alditol acetates and their analysis by GC and combined GC–MS using 3% ECNSS-M and 3% OV-225 columns and a GC–MS system Model 3100 D (Finnigan) were performed as in [13,14].

2.5. Fast atom bombardment and electron impact mass spectrometry

FAB, EI mass spectrometry [15] and ^{1}H NMR [16] were performed as described.

3. RESULTS

The purified glycolipids I (205 μg) and II (195 μg) were homogeneous by criteria of HPTLC,

MS and NMR. Up to 50 μg of the glycolipid fraction containing both components I and II failed to inhibit the agglutination of O Le(a$^-$b$^-$) erythrocytes by both anti-Le^c antisera used here. Also, the same amount did not confer any Le^c antigenicity on O Le^b erythrocytes as tested by passive haemagglutination. When tested against *U. europaeus* lectin, 8 μg glycolipid I completely inhibited 4 haemagglutinating units whereas 20 μg glycolipid II did not effect a complete or even partial inhibition. Analysis of the partially methylated alditol acetates after permethylation, hydrolysis, reduction and acetylation by combined GC–MS furnished for both glycolipids (I and II), in equimolar ratio, derivatives of terminal fucose,
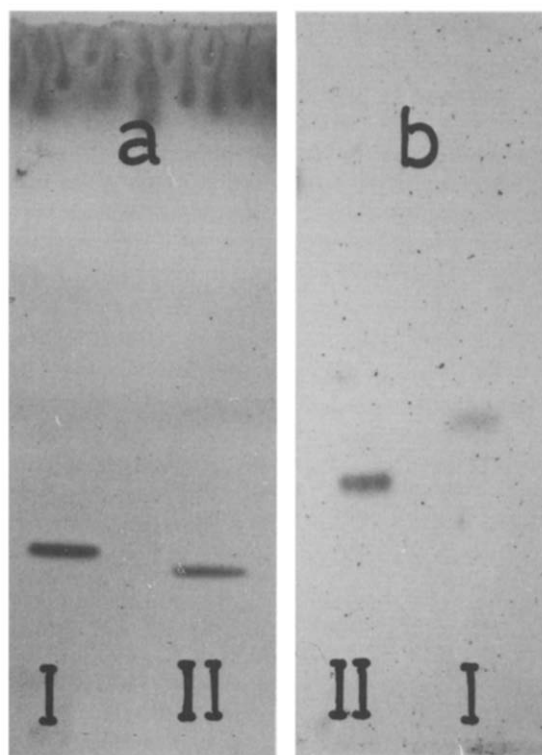

Fig.1. Silica gel high-performance thin-layer chromatography of ceramide pentasaccharides from the plasma of O Le(a$^-$b$^-$) nonsecretors. (a) Native glycolipids (solvent system A) developed in chloroform–methanol–water (60:35:8, v/v). (b) Peracetylated glycolipids developed in 1,2-dichloroethane–methanol–water (97:3:0.2, v/v). Lanes: (1,3) glycolipid I, i.e., lacto-*N*-fucopentaosyl(IV)ceramide; (2,4) glycolipid II, i.e., lacto-*N*-fucopentaosyl(III) ceramide.

3-*O*-substituted galactose and 4-*O*-substituted glucose. Both substances differed however, from each other in the presence of 1 mol of a 2-*O*-substituted galactose and a 4-*O*-substituted *N*-acetylglucosamine in the case of glycolipid I, and a terminal galactose and a 3,4-di-*O*-substituted *N*-acetylglucosamine in the case of glycolipid II. Further evidence for these structures was obtained by mass spectrometry and high-resolution ^{1}H NMR. In agreement with the structure of a ceramide pentasaccharide, the FAB MS of both permethylated glycolipids showed intense pseudomolecular ions M + H$^+$ at 1611 amu and M + Na$^+$ at 1633 amu. In both cases, the ceramide residue was represented by an ion at 548 amu. The terminal tri- and tetrasaccharide units gave rise to ions at *m/z* 638 and 842, but with different intensities and with a different set of daughter ions. The structural differen-

ces between both glycolipids were reflected more markedly in the EI MS spectra as shown in fig.2a,b. In both spectra, the palmitic acid present in the ceramide residue is represented by *m/z* 294 and the sphingosine by *m/z* 364. The major ions derived from the carbohydrate moiety are in perfect agreement with the proposed structures as indicated in the schemes of fragmentation. The daughter ions *m/z* 182 in glycolipid I and *m/z* 432 in glycolipid II clearly indicate the presence of type 2 chains in both compounds [17]. The different site of linkage of the fucose residue gives rise to additional specific rearrangement ions such as *m/z* 1465 in (I) or *m/z* 432 and 1169 in (II).

Using NMR, we have shown that the linkage and sequence analysis of peracetylated oligosaccharides [16,18] and glycosphingolipids [19] is a straightforward procedure thanks to the large up-
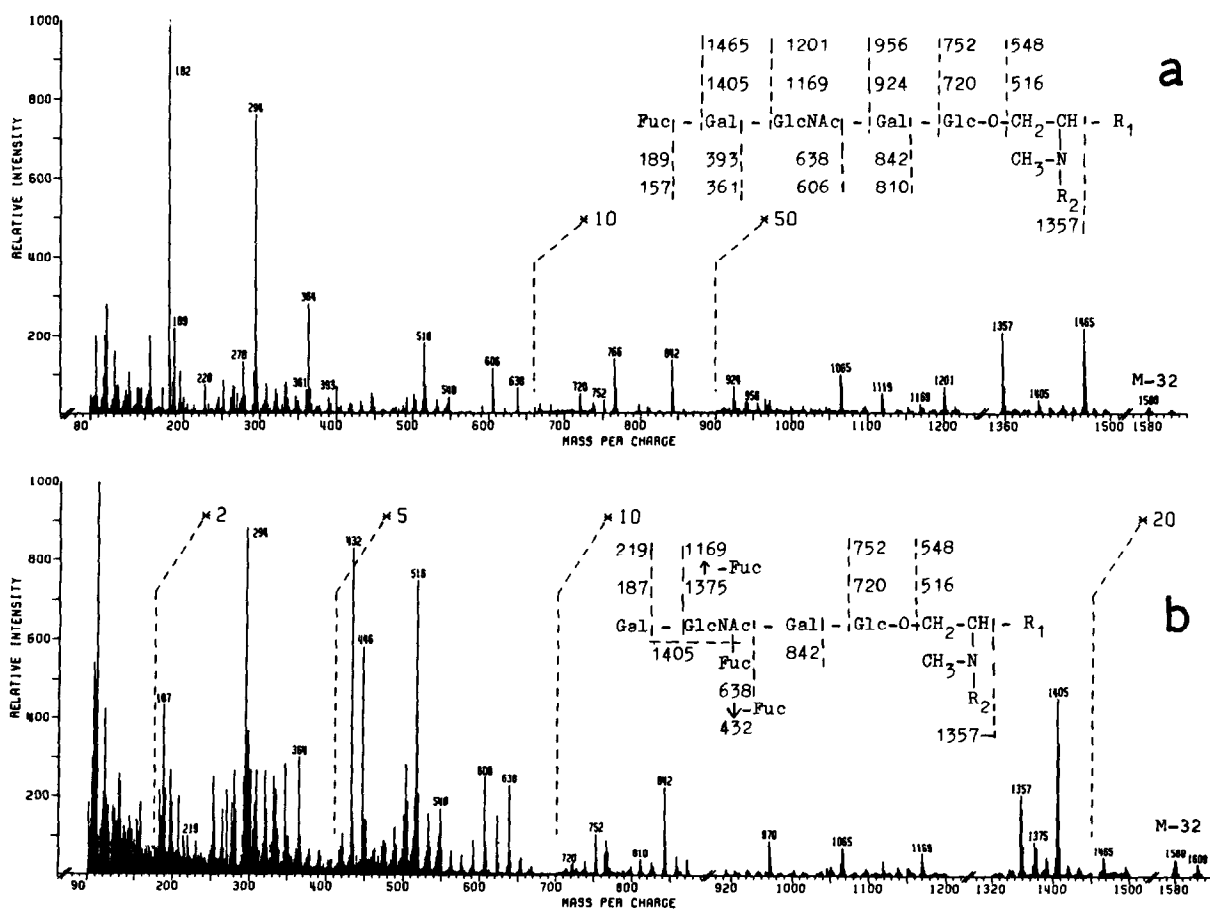

Fig.2. Electron impact mass spectra of permethylated glycolipids I and II. Ionization energy, 25 eV; accelerating voltage, 8 kV; sample and ion source temperature, 270°C.

field chemical shifts observed for protons at glycosylated carbons vs those at acetoxylated ones. Spin-decoupling difference spectra showed that in glycolipid I the H-2 signal of Gal-4 occurs at 3.84 ppm (fig.3b) whereas that of Gal-2 is located at 5.00 ppm (fig.3c). Therefore, fucose is obviously 1-2 linked to Gal-4.

It can be demonstrated in a similar manner that it is the GlcNAc residue in glycolipid II (fig.3d) that is glycosylated by fucose. The GlcNAc H-3 and H-4 resonances are highfield shifted by 1.21 and 1.25 ppm, respectively, if compared with those of the terminal GlcNAc residue of a related glucosaminyl lactitol (GL; table 1) [16], thus unambiguously indicating the two glycosylation positions. The problem of whether fucose is linked to site 3 and galactose to site 4, or the reverse, can be elucidated by comparing GlcNAc chemical shifts for glycolipid II with those of the two isomeric fucopentaitols, fucopentaose-III [20] and fucopentaose-II [16] (table 1). Most of these chemical shifts for glycolipid II and fucopentaose-III are either identical or differ only slightly whereas a number of values for fucopentaose-II deviate much more. Accordingly, glycolipid II and fucopentaose-III are ascribed the same partial sequence:
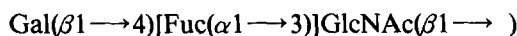
Gal(β1 → 4)[Fuc(α1 → 3)]GlcNAc(β1 →)

Table 1

GlcNAc proton chemical shifts (ppm from Me$_4$Si, in CDCl$_3$) of GL-II, GL[b], FP-III[c] and FP-II[d]

	GL-II	GL	FP-III	FP-II
H-1	4.96	5.05	4.99	4.97
H-2	3.05	3.30	3.05	3.05
H-3	4.29	5.50	4.30	4.37
H-4	3.80	5.05	3.79	3.74
H-5	3.43	3.68	3.43	3.50
H-6	3.94	4.08	3.93	3.74
H-6'	4.99	4.36	4.94	4.80
N-H	5.32	5.38	5.41	5.53

[a] Peracetylated   Gal(β1 → 4)[Fuc(α1 → 3)]GlcNAc-(β1 → 3)Gal(β1 → 4)Glc(β1 → 1)Cer

[b] Peracetylated   GlcNAc(β1 → 3)Gal(β1 → 4)Glc-ol. Data from [16]

[c] Peracetylated   Gal(β1 → 4)[Fuc(α1 → 3)]GlcNAc-(β1 → 3)Gal(β1 → 4)Glc-ol. Data from [20]

[d] Peracetylated   Gal(β1 → 3)[Fuc(α1 → 4)]GlcNAc-(β1 → 3)Gal(β1 → 4)Glc-ol. Data from [16]
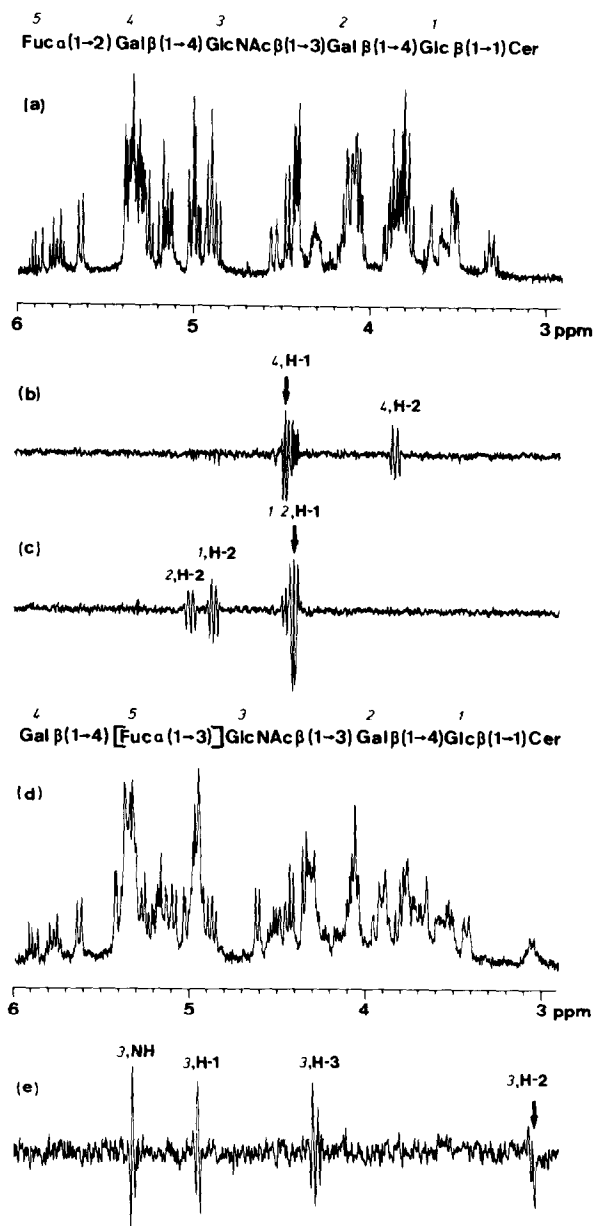

Fig.3. The sugar proton region of the resolution-enhanced 360 MHz ^{1}H NMR spectra of the ceramide fucopentaoses I (a) and II (d), and of the spin-decoupling difference spectra (b,c,e) referred to in the text (irradiation by a second radiofrequency field is indicated by arrows).

All these results are in accordance with a blood-group H type 2 active lacto-N-fucopentaosyl (IV) ceramide substance I and a lacto-N-fucopentaosyl (III) ceramide for substance II.

4. DISCUSSION

Our first objective was to confirm that glycolipids comprising up to 5 sugar units do not represent the molecular basis of the Le^c antigenicity. Instead, traces of more complex, neutral glycosphingolipids isolated simultaneously from the same plasma source showed very strong inhibitory properties of both Le^c and anti-sera[2]. In contrast to Le^c, which is characteristically found only in the plasma of nonsecretors [21], the biosynthesis of the identified structures is obviously independent of the secretor status of an individual [22,23]. Furthermore, the presence of H type 1 (Le^{dH}) ceramide pentasaccharide in the plasma of blood-group O, Lewis negative nonsecretors could also be excluded by physicochemical means. It can be concluded that, in contrast to H type 1 structures, at least a part of the plasmatic H type 2 antigens can be synthesized independently of the influence of the secretor gene. Previous determinations by radioimmunoassay of H type 1 and 2 blood-group activities of human sera from different secretor phenotypes confirm our findings (22). This paper additionally reveals that the secretor gene-independent H type 2 antigenicity of plasma has, at least in part, to be ascribed to glycosphingolipids.

Lacto-*N*-fucopentaosyl (III) structures have been designated as the Le^x antigen [24]. However, anti-Le^x does not react with red cells of all Le(a$^-$b$^-$) individuals [25]. Furthermore, the Le^x property is rather based on a particular specificity of the anti-Le^x antibodies reacting with the Fuc(α1$\longrightarrow$4)GlcNAc region of both the Le^a and Le^b antigenic determinants [26]. Thus, it is suggested to return to the former designation of the 'X-antigenic' determinant for all Gal(β1$\longrightarrow$4) [Fuc(α1$\longrightarrow$3)]GlcNAc residues. The X-antigen has originally been regarded as a tumor-associated cell surface marker [27]. In fact, many antibodies produced by hybridomas obtained from mice immunized with human adenocarcinomas of colon, stomach, or lung are directed against a sugar sequence found in lacto-*N*-fucopentaose III [28]. However, analogous structures also occur in noncancerous tissues or body fluids of healthy individuals, such as human erythrocytes [24], granulocytes and granulocyte precursor cells [28] or in human milk [23], irrespective of the secretor or nonsecretor status of the individual from whom the substances have been isolated. This paper reveals its additional occurrence in human plasma although in minor amounts. Further studies on the quantity of this structure in different human tissues and sources are necessary to define more exactly the behaviour of this antigen during development, and differentiation as has been done for the analogous stage specific embryonic SSEA-1 antigen in mice [30].

ACKNOWLEDGEMENTS

REFERENCES

[1] Hanfland, P. (1978) Eur. J. Biochem. 87, 161–170.
[2] Hanfland, P., Kladetzky, R.-G. and Egli, H. (1978) Chem. Phys. Lipids 22, 141–151.
[3] Hanfland, P. and Graham, H.A. (1981) Arch. Biochem. Biophys. 210, 383–395.
[4] Egge, H. and Hanfland, P. (1981) Arch. Biochem. Biophys. 210, 396–404.
[5] Dabrowski, J., Hanfland, P., Egge, H. and Dabrowski, U. (1981) Arch. Biochem. Biophys. 210, 405–411.
[6] Gunson, H.H. and Latham, V. (1972) Vox Sang. 22, 344–353.
[7] Graham, H.A., Hirsch, H.F. and Davies, D.M. (1977) in: Human Blood Groups (Mohn, J.F. et al. eds) pp.257–267, Karger, Basel.
[8] Ginsburg, V., McGinnis, M.H. and Zopf, D.A. (1980) Prog. Clin. Biol. Res. 43, 45–53.
[9] Le Pendu, J., Lemieux, R.U. and Oriol, R. (1982) Vox Sang. 43, 188–195.
[10] Hanfland, P., Graham, H.A., Crawford, R.J. and Schenkel-Brunner, H. (1982) FEBS Lett. 142, 77–80.
[11] Hanfland, P., Egge, H., Dabrowski, U., Kuhn, S., Roelcke, D. and Dabrowski, J. (1981) Biochemistry 20, 5310–5319.
[12] Hanfland, P. and Egli, H. (1975) Vox Sang. 28, 438–452.
[13] Stoffel, W. and Hanfland, P. (1973) Hoppe Seyler's Z. Physiol. Chem. 354, 21–32.
[14] Hanfland, P. (1975) Chem. Phys. Lipids 15, 105–124.

[15] Egge, H., Peter-Katalinić, J., Paz-Parente, J., Strecker, G., Montreuil, J. and Fournet, B. (1983) FEBS Lett. 156, 357–362.

[16] Dabrowski, U., Egge, H. and Dabrowski, J. (1983) Arch. Biochem. Biophys. 224, 254–260.

[17] Egge, H. (1978) Chem. Phys. Lipids 21, 349–360.

[18] Dabrowski, J., Egge, H. and Dabrowski, U. (1983) Carbohydr. Res. 114, 1–9.

[19] Dabrowski, J., Hanfland, P., Kordowicz, M., Egge, H., Dabrowski, U. and Hull, W.E. (1984) Eur. J. Biochem., submitted.

[20] Egge, H., Dabrowski, U. and Dabrowski, J., unpublished.

[21] Hirsch, H.F. and Graham, H.A. (1980) Transfusion 20, 474–475.

[22] Le Pendu, J., Lemieux, R.U., Lambert, F., Dalix, A.-M. and Oriol, R. (1982) Am. J. Hum. Genet. 34, 402–415.

[23] Kobata, A. and Ginsburg, V. (1969) J. Biol. Chem. 224, 5496–5502.

[24] Kannagi, R., Nudelman, E., Leroy, S.B. and Hakomori, S.-I. (1982) J. Biol. Chem. 257, 14865–14874.

[25] Arcilla, M.B. and Sturgeon, P. (1974) Vox Sang. 26, 425–438.

[26] Schenkel-Brunner, H. and Hanfland, P. (1981) Vox Sang. 40, 358–366.

[27] Yang, H.-A. and Hakomori, S.-I. (1971) J. Biol. Chem. 246, 1192–1200.

[28] Huang, L.C., Brockhaus, M., Magnani, J.L., Cuttitta, F., Rosen, S., Minna, J.D. and Ginsburg, V. (1983) Arch. Biochem. Biophys. 220, 318–320.

[29] Huang, L.C., Civin, C.I., Magnani, J.L., Shaper, J.H. and Ginsburg, V. (1983) Blood 61, 1020–1023.

[30] Gooi, H.C., Feizi, T., Kapadia, A., Knowles, B.B., Solter, D. and Evans, M.J. (1981) Nature 292, 156–158.