J. Am. Chem. Soc. 1988, 110, 4409-4411

Enzymes in Organic Synthesis. 42.[1] Investigation of the Effects of the Isozymal Composition of Pig Liver Esterase on Its Stereoselectivity in Preparative-Scale Ester Hydrolyses of Asymmetric Synthetic Value[2]

Lister K. P. Lam, Christine M. Brown, Bernard De Jeso,† Laura Lym, Eric J. Toone, and J. Bryan Jones*

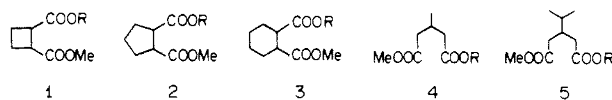
Contribution from the Department of Chemistry, University of Toronto, Toronto, Ontario M5S 1A1, Canada. *Received December 7, 1987*

Abstract: The stereospecificities of the isozyme components of commercially available pig liver esterase have been shown to be essentially the same toward representative monocyclic and acyclic diester substrates. This removes previous concerns that the isozymal composition of pig liver esterase, a widely used catalyst for chiral synthon production, might result in its not behaving consistently when applied as a catalyst for asymmetric synthetic purposes. The results establish that commercial pig liver esterase can be exploited synthetically as a chiral catalyst with confidence that it will behave as if it were a single protein.

Enzymes are now widely exploited as catalysts in asymmetric syntheses.[3] Esterases are among the most attractive enzymes in this regard because they operate on their ester substrates without requiring expensive coenzymes. Pig liver esterase (PLE, EC 3.1.1.1) is one of this group of hydrolases that has already proven of considerable value for the generation of useful chiral synthons. The potential of PLE as a valuable catalyst in asymmetric synthesis remains considerable, and it continues to receive widespread attention.[4-6]

One of the interesting aspects of the specificity of PLE in its catalysis of hydrolysis of diesters is that a number of structure-induced reversals of stereoselectivity have been reported in both the monocyclic[5] and acyclic[5a,6] series of substrates. While other reversals of enzyme stereospecificity within structurally related substrate series are known,[8] they are not common. Furthermore, unless the basis for such reversals can be established, such uncertainties constitute a severe disadvantage for any enzyme that is a candidate for general application in asymmetric synthesis.

The commercially available PLE preparations used synthetically are mixtures of isozymes.[9] Consequently, we felt that a possible explanation for the various stereoselectivity reversals reported[5,6] for the commercial enzyme was that different isozymes might have different stereospecifities, with whichever isozyme(s) interacting best with a given substrate structure controlling the overall stereochemical outcome. We therefore separated commercial PLE into its major isozyme fractions and examined the enantiotopic ester group stereospecificity of each with respect to hydrolyses of the diester substrates **1b–5b** for which reversals of enantiotopic



stereoselectivity have been documented.[5d,6c] The results obtained show that all the isozymes have fundamentally the same stereospecificity and that the commercially available PLE enzyme mixture can therefore be used with confidence in asymmetric synthetic applications as if it were a single species.

Results

The diester substrates **1b–5b** were prepared as described previously.[5d,6c]

Commercial PLE was separated into its major isozyme fractions by isoelectric focusing according to the procedure of Heymann and Junge.[9c] The fractions were assayed with phenyl acetate as substrate.[9a] The enzyme activity pattern observed, shown in Figure

Table I. Relative Rates of Hydrolyses of **1b** and **3b–5b** Catalyzed by Isozyme Fractions A–F[a]

	rel rate,[b] %					
substr	A	B	C	D	E	F
1b	9	9	17	24	23	42
3b	5	5	6	8	10	13
4b	54	44	49	100	87	68
5b	3	2	2	4	3	2

[a] Determined titrimetrically with 0.01 or 0.02 M aqueous NaOH using a pH-stat, [S] = 0.5 mM (for **1b** and **3b**) or 4 mM (for **4b** and **5b**), 5% aqueous MeOH, 0.03 M KH_2PO_4 (pH 7), 25 °C, 0.5 mL of isozyme solution. Not determined for **2b**. [b] All relative to **4b**-D = 100.

† Present address: Laboratory of Organic and Organometallic Chemistry, University of Bordeaux I, Talence Cedex, France.

(1) Part 41: Lam, K. P. L.; Gair, I. A.; Jones, J. B., submitted for publication in *J. Org. Chem.*

(2) Abstracted in part from: Lam, K. P. L. Ph.D. Thesis, University of Toronto, 1986.

(3) (a) Jones, J. B.; Beck, J. F. *Tech. Chem. (N.Y.)* **1976**, *10*, 107. Jones, J. B. *Tetrahedron* **1986**, *42*, 3351. (b) Wong, C. H.; Whitesides, G. M. *Angew. Chem., Int. Ed. Engl.* **1985**, *24*, 617. Akiyama, A.; Bednarski, M.; Kim, M.-J.; Simon, E. S.; Waldman, H.; Whitesides, G. M. *Chem. Br.* **1987**, *23*, 645. (c) Klibanov, A. M. *Science (Washington, D.C.)* **1983**, *219*, 722; *CHEMTECH* **1986**, 354. (d) *Enzymes Org. Synth., Ciba Found. Symp.* **1985**, *111*. (e) Tramper, J., von der Plas, H. C., Linko, P., Eds. *Biocatalysis in Organic Synthesis*; Elsevier: Amsterdam, 1985. (f) Schneider, M., Ed. *Enzymes as Catalysts in Organic Synthesis*; Reidel: Dordrecht, The Netherlands, 1986.

(4) (a) Sicsic, S.; LeRoy, J.; Wakselman, C. *Synthesis* **1987**, 155. (b) Riva, R.; Banfi, L.; Danieli, B.; Guanti, G.; Lesma, G.; Palmisano, G. *J. Chem. Soc., Chem. Commun.* **1987**, 299. (c) Kamiyama, K.; Kobayashi, S.; Ohno, M. *Chem. Lett.* **1987**, 29. (d) Klunder, A. J. H.; Huizinga, W. B.; Sessnik, P. J. M.; Zwanenburg, B. *Tetrahedron Lett.* **1987**, *28*, 357. (e) Mohr, P.; Roesslein, L.; Tamm, C. *Helv. Chim. Acta* **1987**, *70*, 142. (f) Bjorkling, F.; Boutelje, J.; Hjalmarsson, M.; Hult, K.; Norin, T. *J. Chem. Soc., Chem. Commun.* **1987**, 1041.

(5) (a) Mohr, P.; Waespe-Sarcevic, N.; Tamm, C. *Helv. Chim. Acta* **1983**, *66*, 2501. (b) Schneider, M.; Engel, N.; Honicke, P.; Heinesmann, G.; Gorisch, H. *Angew. Chem., Int. Ed. Engl.* **1984**, *23*, 67. (c) Gais, H. J.; Lukas, K. L. *Angew. Chem., Int. Ed. Engl.* **1984**, *23*, 142. (d) Sabbioni, G.; Shea, M. L.; Jones, J. B. *J. Chem. Soc., Chem. Commun.* **1984**, 236. Sabbioni, G.; Jones, J. B. *J. Org. Chem.* **1987**, *52*, 4565. (e) Jones, J. B.; Hinks, R. S.; Hultin, P. G. *Can. J. Chem.* **1985**, *63*, 452.

(6) (a) Ohno, M.; Kobayashi, S.; Iimori, T.; Wang, Y. F.; Izawa, T. *J. Am. Chem. Soc.* **1981**, *103*, 2405. Wang, Y. F.; Izawa, T.; Kobayashi, S.; Ohno, M. *J. Am. Chem. Soc.* **1982**, *104*, 6465. (b) Bjorkling, F.; Boutelje, J.; Gatenbeck, S.; Hult, K.; Norin, T.; Szmulik, P. *Tetrahedron* **1985**, *41*, 1347. (c) Lam, L. K. P.; Hui, R. A. H. F.; Jones, J. B. *J. Org. Chem.* **1986**, *51*, 2047.

(7) In the context of this paper, the term reversal of stereoselectivity refers to, for example, an initial *pro-R* ester group hydrolysis changing over to a *pro-S* preference within the same series of *meso*-diester substrate structures.

(8) (a) Schwartz, H. M.; Wu, W.-S.; Marr, P. W.; Jones, J. B. *J. Am. Chem. Soc.* **1978**, *100*, 5199. (b) Keinan, E.; Hafeli, E. K.; Seth, K. K.; Lamed, R. *J. Am. Chem. Soc.* **1986**, *108*, 162. (c) Zaks, A.; Klibanov, A. M. *J. Am. Chem. Soc.* **1986**, *108*, 2767.

0002-7863/88/1510-4409$01.50/0 © 1988 American Chemical Society

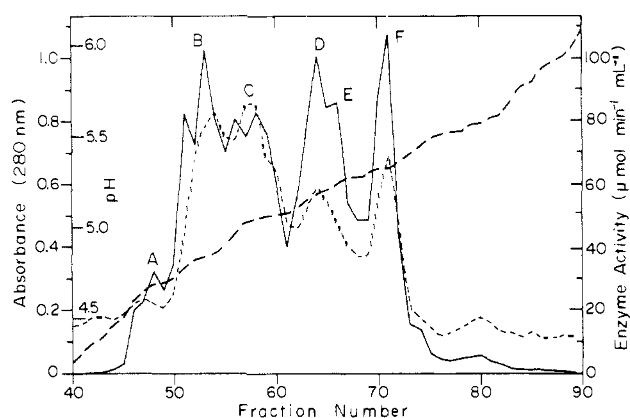
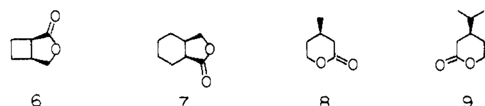


Figure 1. Assay of isozyme fractions of pig liver esterase: (—) enzyme activity; (- - -) absorbance (280 nm) as a measure of protein concentration; (– –) pH gradient. A–F represent the fractions used in the preparative-scale experiments.

1, is in accordance with that reported by Farb and Jencks.[9a] For the preparative-scale reactions, the individual enzyme fractions from isoelectric focusing were combined into six major isozyme solutions A–F, with the activity minima separating the maxima being the cutoff points between the pooled fractions.[10] The protein concentrations of each of fractions A–F were approximately equal, being in the 290–400 μg/mL range.[11] Their activities toward the substrates **1b–5b** varied somewhat. The relative activities observed for **1b** and **3b–5b** are recorded in Table I.

The preparative-scale hydrolyses of diesters **1b–5b** were then evaluated with each of the PLE isozyme solutions A–F. The ee's of the acid ester products **1a–5a** were determined by ^{1}H NMR analysis in the presence of (+)-(*R*)-2-phenylethylamine[5b] for **1a** and **2a** and via lactone **7** for **3a**[12] and by the GLC ortho ester



analytical method[13] for the lactones **8** or **9** derived from **4a** and **5a**, respectively. The absolute configurations of the acid esters **1a** and **3a–5a** were established from the known configurations of their lactone derivatives **6–9** as described previously[5d,6c] and those of **2a** from the known[5d] optical rotation. The results are summarized in Table II.

Discussion

The amounts of isozyme-fraction material needed for the Table II preparative-scale experiments required several separate fractionations of PLE by isoelectric focusing. In all cases, the separations gave six major isozyme fractions (Figure 1) within the p*I* range of activity of (4.8–5.7) ± 0.1.

The relative rate comparisons shown in Table I show that there are significant differences in the activities of the isozymes toward the different substrates, with the less bulky diesters being the more active in both the cyclic and acyclic substrate series. These assays were performed in 5% aqueous methanol in order to ensure full substrate solubility. However, it must be noted that methanol is not an innocuous cosolvent. We confirmed its acceleration of phenyl acetate hydrolysis[9a] and observed 4-fold activity decreases over the purely aqueous assays with ethyl butyrate as substrate. In both cases, the rate changes are due to interception of the acyl-enzyme intermediate by the methanol.[9a] The behaviors of diesters **1b–5b** with each isozyme fraction are analogous to that of ethyl butyrate. Hence, the activities of the isozyme fractions used in the wholly aqueous preparative-scale conditions will be somewhat higher than indicated by the Table I data. Nevertheless, the differences in the preparative reaction times observed (1.5–80 h) still vary by the same ~50-fold factor observed in the range of the Table I rates.

The results summarized in Table II show clearly that the stereospecificities of each of the different isozyme fractions A–F are substantially the same. For the substrates **1b** and **3b** that are hydrolyzed with high stereoselectivity to give acid esters of very high, or complete, enantiomeric purities, the stereospecificity variations between different isozyme runs are either nonexistent or minor. For **4b** and **5b**, where the enzymic enantiotopic selectivities are less complete, the ee variations between isozyme runs are again within the combined error limits of the experimental and ee determination methods. This observation is of general validity despite the fact that the enantiotopic selectivity of PLE is opposite for **1b**, **3b** and **4b**, **5b**. The same holds true for isozymal hydrolyses of the cyclopentane diester **2b**, which, being the reversal-of-stereospecificity-point substrate within the **1b–3b** cycloalkane diester series, is an excellent test case. For this substrate, the range of ee differences observed with isozymal fractions A–F is somewhat greater than for the corresponding hydrolyses of **1b** and **3b–5b**. However, once again, the overall data confirm that, as with the whole enzyme, no isozyme fraction is capable of high stereoselectivity in catalyzing hydrolyses of **2b**.

Table II. Hydrolyses of Diesters **1b-5b** Catalyzed by Isozyme Fractions A–F[a]

substr	isozyme fractn	product	yield,[b] % (% ee)
1b	A	(1*R*,2*S*)-**1a**	89 (95)
1b	B	(1*R*,2*S*)-**1a**	86 (97)
1b	C	(1*R*,2*S*)-**1a**	90 (95)
1b	D	(1*R*,2*S*)-**1a**	92 (>97)
1b	E	(1*R*,2*S*)-**1a**	90 (>97)
1b	F	(1*R*,2*S*)-**1a**	92 (>97)
1b	whole PLE	(1*R*,2*S*)-**1a**	87 (>97)[5d]
2b	A	(1*S*,2*R*)-**2a**	*c* (13)
2b	B	(1*S*,2*R*)-**2a**	80 (8)
2b	C	(1*S*,2*R*)-**2a**	78 (4)
2b	D	(1*S*,2*R*)-**2a**	80 (1)
2b	E	(1*S*,2*R*)-**2a**	87 (1)
2b	F	(1*S*,2*R*)-**2a**	50[d] (12)
2b	whole PLE	(1*S*,2*R*)-**2a**	92 (17)[5d]
3b	A	(1*S*,2*R*)-**3a**	92 (>97)
3b	B	(1*S*,2*R*)-**3a**	92 (>97)
3b	C	(1*S*,2*R*)-**3a**	92 (>97)
3b	D	(1*S*,2*R*)-**3a**	97 (>97)
3b	E	(1*S*,2*R*)-**3a**	82 (>97)
3b	F	(1*S*,2*R*)-**3a**	97 (>97)
3b	whole PLE	(1*S*,2*R*)-**3a**	91 (>97)
4b	A	(3*R*)-**4a**	95 (63)
4b	B	(3*R*)-**4a**	92 (69)
4b	C	(3*R*)-**4a**	89 (80)
4b	D	(3*R*)-**4a**	67 (75)
4b	E	(3*R*)-**4a**	99 (77)
4b	F	(3*R*)-**4a**	94 (78)
4b	whole PLE	(3*R*)-**4a**	94 (79)[6c]
5b	A	(3*S*)-**5a**	69 (17)
5b	B	(3*S*)-**5a**	93 (17)
5b	C	(3*S*)-**5a**	72 (22)
5b	D	(3*S*)-**5a**	82 (30)
5b	E	(3*S*)-**5a**	94 (26)
5b	F	(3*S*)-**5a**	*c* (16)
5b	whole PLE	(3*S*)-**5a**	99 (38)[6c]

[a] In aqueous 0.001–0.03 M KH_2PO_4 (pH7), 0.1 M KCl, 20 °C. [b] After Kugelrohr distillation. [c] Quantitative from base takeup, isolated yield not recorded. [d] Low due to incomplete ether extraction step.

(9) (a) Farb, D.; Jencks, W. P. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **1974**, *33*, 1477; *Arch. Biochem. Biophys.* **1980**, *203*, 214. (b) Hamilton, S. E.; Campbell, H. D.; de Jersey, J.; Zerner, B. *Biochem. Biophys. Res. Commun.* **1975**, *63*, 1146. (c) Heymann, E.; Junge, W. *Eur. J. Biochem.* **1979**, *95*, 509.

(10) The median p*I* values of the pooled fractions A–F are indicated in Figure 1. In repeated isoelectric focusing experiments, while the basic enzyme activity profile remained fundamentally the same, the p*I* values of the A–F maxima varied by ±0.2 unit due to differences in the pH gradients applied. Solutions A–F do not correspond to fractions I–IV of Heymann and Junge.[9c]

(11) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265.
(12) Jakovac, I. J.; Jones, J. B. *J. Org. Chem.* **1979**, *44*, 2165.
(13) Saucy, G.; Borer, R.; Trullinger, D. P.; Jones, J. B.; Lok, K. P. *J. Org. Chem.* **1977**, *42*, 3206.

The fact that the isozymal composition of commercial PLE does not affect the overall stereoselectivity of its catalysis of the hydrolyses of several dissimilar substrates of two different structural series was an unexpected discovery. However, it is one that is of considerable asymmetric importance, since it means that in asymmetric synthetic applications commercial PLE can be used with confidence as if it were a single protein.

Experimental Section

The instrumentation and general purification and analytical procedures used were as described previously.[5d,6c] Pig liver esterase (PLE, EC 3.1.1.1) was Sigma Chemical Co. type II (lot 123F-0240). The diester substrates **1b–5b** were prepared by the literature methods.[5d,6c]

PLE Isozyme Separation. The basic method of Heymann and Junge[9c] and of Farb and Jencks[9a] was used, with minor modifications as follows:

Isoelectric focusing of PLE in a sucrose gradient (0–70%) was carried out at 400 V for 48 h at 4 °C with a 440-mL column using 10 mL of Ampholine (pH 4–6) and 4.4 mL of Ampholine (pH 5–7) (LKB) with 2% ethylenediamine at the cathode and 1% (v/v) phosphoric acid in 86% sucrose at the anode. The pig liver esterase suspension (40 mg in 4 mL) was dialyzed against doubly distilled deionized water for 48 h before use. The dialyzed enzyme was added when approximately half of a linear sucrose gradient had been put into the column. The pHs of the isolated fractions (160 × 3 mL) were measured and neutralized with 1 M dipotassium phosphate before the enzyme activities toward different substrates were assayed.

Protein concentrations were determined from the absorbance at 280 nm on the basis of an extinction coefficient of $\epsilon_{1cm}^{1\%}$ 13.8.[14] Esterase activities of each fraction were assayed spectrophotometrically by the method of Farb and Jencks[9a] with phenyl acetate (3 mM) in 0.03 M KH_2PO_4 (pH 7.0) at 25 °C. A representative assay is shown in Figure 1.

For the preparative-scale experiments, isozyme solutions A–F were created by combining the fractions centered around the activity peaks at p*I* 4.70, 4.96, 5.06, 5.19, 5.24, and 5.38, respectively. The protein concentrations of these solutions were determined by the Lowry method[11] (Sigma protein assay kit P5656) to be 400, 290, 320, 390, 315, and 355 μg/mL, respectively. The relative rates of hydrolyses of **1b** and **3b–5b** by A–F were determined in the usual way, using a pH-stat.[5e] The results are recorded in Table I.

Preparative-Scale Isozyme-Catalyzed Hydrolyses of 1b–5b. The experimental procedures followed, and ee and absolute configuration methods used, were as described previously for the whole PLE studies.[5d,6c] The reactions were carried out on 200–500 mg of **1b–5b** with ~0.4–1 mg of each isozyme group (~1–3 mL, still containing ampholytes and sucrose) under the usual[5d,6c] pH 7 conditions at 20 °C. The reaction times varied from 1.5–80 h, with the individual times being roughly proportional to the relative rate orders of Table I. The preparative-scale results are summarized in Table II.

The acid ester products **1a–5a** obtained, and their lactone **6–9** derivatives, were identical in respect to physical properties and spectra with the previous samples[5d,6c] except for the magnitudes of their optical rotations. The $[\alpha]^{25}_D$ values (in $CHCl_3$ unless specified otherwise) observed for the acid ester product of each ester substrate–isozyme reaction combination were as follows.

1a: A –3.56° (*c* 4.2); B, –3.56° (*c* 4.2); C, –2.10° (*c* 2.1); D, –3.08° (*c* 6.0); E, –2.40° (*c* 1.5); F, –2.73° (*c* 2.2); whole PLE, –3.0° (*c* 2.1) for >97% ee.[6c]

2a: A, +0.84° (*c* 3.95); B, +0.76° (*c* 3.95); C, +0.26° (*c* 2.7); D, +0.35° (*c* 2.4); E, +0.44° (*c* 2.3); F, +0.83° (*c* 4.0); whole PLE, +1.0° (*c* 1) for 17% ee.[5d]

3a: A, –0.53° (*c* 6.1);[15] B, –0.78 (*c* 4.5); C, –0.75° (*c* 4.0); D, –0.96° (*c* 10.0); E, –0.51° (*c* 1.95); F, –0.96° (*c* 8.9); whole PLE, –1.26° (*c* 1.8)[15] (lit.[5d] $[\alpha]^{25}_D$ +4.7° (*c* 0.87, $CHCl_3$).

4a: Rotations determined on derived lactones.

(4*R*)-**8a**: A, +16.90° (*c* 1.4); B, +19.25° (*c* 4.0); C, +19.63° (*c* 0.55); D, +18.06° (*c* 3.14); E, +20.90° (*c* 8.4), F, +19.55° (*c* 4.0); whole PLE, +21.7° (*c* 4) for 79% ee.[6c]

5a: A, –1.93° (*c* 1.0); B, –4.02° (*c* 1.5); C, –5.43° (*c* 1.4); D, –8.80° (*c* 1.2); E, –6.78° (*c* 1.7); F, –5.9° (*c* 3.1); whole PLE –11.1° (*c* 7.3) for 38% ee.[6c]

Enantiomeric Excess and Absolute Configuration Determinations. The ee's of the acid esters **1a–5a** were determined as previously reported,[5d,6c] as follows: of **1a** and **2a** by ^{1}H NMR analysis in the presence of (+)-(2*R*)-phenylethylamine[5b] and of **3a** by ^{1}H NMR analysis on its lactone derivative **7**;[12] of **4a** and **5a** by GLC analysis of the ortho esters[13] of their lactone derivatives **8** and **9**. The results are recorded in Table II. The absolute configuration correlations of **1a** and **3a–5a** were with the known[5d,6c] configurations of their lactone derivatives **6–9** and that of **2a** from comparisons of their optical rotations with the literature value.[5d]

Acknowledgment. This work was supported by the Natural Sciences and Engineering Research Council of Canada. We are grateful to NATO for a research grant to B.D.J. and to the University of Toronto for open fellowship awards to L.K.P.L. and E.J.T. and for a Gollop Memorial Scholarship to L.K.P.L. We thank Dr. R. A. H. F. Hui for completing the **5b**-F experiment.

Registry No. (1*R*,2*S*)-**1a**, 88335-89-1; **1b**, 2607-03-6; (1*S*,2*R*)-**2a**, 88315-64-4; **2b**, 4841-91-2; (1*S*,2*R*)-**3a**, 88335-92-6; **3b**, 1687-29-2; (3*R*)-**4a**, 63473-60-9; **4b**, 19013-37-7; (3*S*)-**5a**, 91478-83-0; **5b**, 2338-44-5; EC 3.1.1.1, 9016-18-6.

(14) Barker, D. L.; Jencks, W. P. *Biochemistry* **1969**, *8*, 3879.

(15) The negative rotations were found for all Kugelrohr-distilled material and contrast the positive rotations for (1*S*,2*R*)-**3a** reported previously.[5b,d] Our previous positive rotation[5d] was on undistilled material. It seems that (1*S*,2*R*)-**3a** epimerizes at the ester center during distillation to give the more stable trans-(1*R*,2*R*,) isomer, which has a negative rotation.[16] Rotation change from positive to negative also occurs on keeping solutions of (+)-(1*S*,2*R*)-**3a** (cf., ref 15). This does not affect the ee or absolute configuration assignments of Table II since these were determined on the (1*S*,2*R*)-**7** lactone derivatives, formation of which requires reepimerization of the 1*R* ester center of the *trans*-**3a** acid esters back to 1*S* prior to forming the γ-lactone ring.

(16) Bjorkling, F.; Boutelje, J.; Gatenbeck, S.; Hult, K.; Norin, T. *Appl. Microbiol. Biotechnol.* **1985**, *21*, 16.