Comparison of Stationary Phases in Reversed-Phase TLC for Correlation between Structure and Biological Response of Probiotics

AKIRA FUJII *§, KENNETH E. SHORES ‡¶, JAMES H. BUSH ‡‖, RICHARD J. GARASCIA ‡, and ELTON S. COOK *x

Received March 24, 1977, from the **Division of Chemistry and Biochemistry, St. Thomas Institute, Cincinnati, OH 45206,* and the ‡*Department of Chemistry, Xavier University, Cincinnati, OH 45207.* Accepted for publication August 8, 1977. §Present address: Department of Pharmacology, Nihon University School of Dentistry at Matsudo, 870-1, Sakaecho Nishi-2, Matsudo-shi, Chiba-ken 271, Japan. ¶Present address: Celanese Polymers and Specialties Co., Louisville, Ky. ‖Present address: Department of Chemistry, University of Wyoming, Laramie, Wyo.

Abstract □ A series of C_{16}, C_{18}, and C_{20} fatty acids and their ethyl esters and alcohols were investigated as possible stationary phases in reversed-phase TLC for the correlation between structure and biological response (antistaphylococcal activity). Ten probiotics (ω-amino acids and their L-histidine dipeptides) were used as the biologically active compounds. The mobile phase was 70% acetone in water. The best correlations were obtained with hexadecanoic acid (palmitic acid) or *cis*-9,*cis*-12,*cis*-15-octadecatrienoic acid, 1-hexadecanol or *cis*-9-octadecenol, and ethyl hexadecanoate for the fatty acids, their alcohols, and their ethyl esters, respectively. Among all compounds, the following relation was obtained: fatty acids = alcohols > ethyl esters > white paraffin oil.

Keyphrases □ Fatty acids and derivatives, various—evaluated as stationary phases in reversed-phase TLC for correlation between structure and biological response of probiotics □ TLC, reversed phase—various fatty acids and derivatives evaluated as stationary phases for correlation between structure and biological response of probiotics □ Probiotics, various—ω-amino acids and derivatives, reversed-phase TLC for correlation between structure and biological response, various fatty acids and derivatives evaluated as stationary phases □ ω-Amino acids and derivatives, various—reversed-phase TLC for correlation between structure and biological response, various fatty acids and derivatives evaluated as stationary phases □ Structure–activity relationships—various fatty acids and derivatives evaluated as stationary phases in reversed-phase TLC for correlation between structure and biological response of probiotics

In recent years, there has been increased interest in reversed-phase TLC for the correlation between structure and biological response of a series of probiotics (1, 2), *N*-*n*-alkyltritylamines (3), acridylmethanesulfonanilides (4), penicillin and cephalosporins (5), bis(dichloroacetamides) and vitamin K analogs (6), testosterone esters (7), sulfonamides (8), rifamycin derivatives (9), phenols (10), steroids (11), and benzenesulfonamide pyrimidines (12). An extensive review was prepared by Biagi *et al.* (5). Stationary phases were white paraffin oil (1–4, 12) and silicone[1] (5–11) on silica gel G thin-layer plates.

Since the correlation between structure and biological response depends greatly on the correlation between the physicochemical and biological behavior of the compounds and since the partition coefficient of the compounds is one of the most important physicochemical factors, further investigation of the stationary phase in reversed-phase TLC of probiotics was initiated.

The present investigation was undertaken to improve the correlation between structure and biological response of probiotics by studying a series of C_{16}, C_{18}, and C_{20} fatty acids and their esters and alcohols as stationary phases. These stationary phases were chosen to reproduce the fatty acid components of the cell membrane.

[1] Dow-Corning 200 fluid.

Table I—Stationary Phases

IUPAC Name	Common Name	Concentration, %
Eicosanoic acid	Arachidic acid[a]	0.5 (w/v)
Ethyl eicosanoate	Ethyl arachidate	0.5 (w/v)
cis-9,*cis*-12,*cis*-15-Octadecatrienoic acid	Linolenic acid	1.0 (v/v)
cis-9,*cis*-12-Octadecadienoic acid	Linoleic acid[a]	1.0 (v/v)
cis-9-Octadecenoic acid	Oleic acid	1.0 (v/v)
Octadecanoic acid	Stearic acid	0.5 (w/v)
Ethyl *cis*-9,*cis*-12-octadecadienoate	Ethyl linoleate	1.0 (w/v)
Ethyl *cis*-9-octadecenoate	Ethyl oleate	1.0 (v/v)
Ethyl octadecanoate	Ethyl stearate	0.5 (v/v)
cis-9-Octadecenol	Oleyl alcohol	1.0 (w/v)
1-Octadecanol	1-Octadecanol	0.5 (w/v)
cis-9-Hexadecenoic acid	Palmitoleic acid	1.0 (w/v)
Hexadecanoic acid	Palmitic acid	0.5 (w/v)
Ethyl hexadecanoate	Ethyl palmitate	1.0 (w/v)
1-Hexadecanol	Cetyl alcohol	0.5 (w/v)
—	White paraffin oil	5.0 (v/v)

[a] Eastman Organic Chemicals.

EXPERIMENTAL[2]

As in the previous study (2), the reversed-phase TLC R_m values were obtained by the method described by Boyce and Milborrow (3) using silica gel G TLC plates[3] (10 × 20 cm) coated with a 250-μm layer of silica gel G. After activation at 105° for 10 min, the plates were impregnated by allowing a reversed-phase compound solution in ether, except white paraffin oil which was dissolved in hexane, to cover the plate; the solvent was then evaporated at 40°. White paraffin oil, used previously (1, 2), served as the positive control. The other phases and the concentrations are summarized in Table I.

ω-Amino acids (glycine, β-alanine, γ-aminobutyric acid, δ-aminovaleric acid, and ε-aminohexanoic acid) and ω-aminoacyl-L-histidines (glycyl-L-histidine, β-alanyl-L-histidine, γ-aminobutyryl-L-histidine, δ-aminovaleryl-L-histidine, and ε-aminohexanoyl-L-histidine), which possess antistaphylococcal (probiotic) activity (13), were used as biologically active compounds. Biological response, defined as percent protection against mortality from staphylococcal infections in mice, was previously reported (13). L-Alanine was used as a control for reversed-phase TLC.

Solutions containing 1% of the compounds in 10% (v/v) 2-propanol were spotted and then developed[4] with 70% acetone in water (v/v), previously determined to be optimal (2). The spots were located by the ninhydrin reaction. The R_m values were calculated from:

$$R_m = \log(1/R_f - 1) \qquad \text{(Eq. 1)}$$

The R_m values were correlated with the biological response to the probiotics by computer[5] regression analysis. The program[6] was used to obtain least-squares fits for the data in first-, second-, and third-order

[2] Solvents and chemicals, except where indicated, were purchased from Fisher Scientific and Matheson, Coleman and Bell.
[3] Analtech, Inc.
[4] Polyethylene chamber, Analtech, Inc.
[5] IBM.
[6] Developed by the Statistics Department of Pennsylvania State University.

Table II—Regression Analysis (Antistaphylococcal Activity, Y) = $aX + b$ ($X = R_m$)

Compound	a	b	n	r	s
Hexadecanoic acid	77.6	30.0	10	0.944	9.19
cis-9-Hexadecenoic acid	83.5	11.3	10	0.909	11.60
Octadecanoic acid	67.9	40.7	10	0.854	14.50
cis-9-Octadecenoic acid	73.6	25.2	10	0.940	9.50
cis-9,*cis*-12-Octadecadienoic acid	70.6	38.2	10	0.929	10.30
cis-9,*cis*-12,*cis*-15-Octadecatrienoic acid	78.9	5.8	10	0.945	9.12
Eicosanoic acid	78.7	27.4	10	0.877	10.10
Ethyl hexadecanoate	107.0	19.7	10	0.929	10.30
Ethyl octadecanoate	109.0	10.2	10	0.921	10.90
Ethyl *cis*-9-octadecenoate	99.6	5.8	10	0.920	10.90
Ethyl *cis*-9,*cis*-12-octadecadienoate	98.0	−5.8	10	0.918	11.10
Ethyl eicosanoate	199.0	−11.8	10	0.880	13.20
1-Hexadecanol	109.0	14.7	10	0.933	10.00
1-Octadecanol	108.0	4.4	10	0.930	10.30
cis-9-Octadecenol	97.7	13.7	10	0.933	10.10
White paraffin oil	92.7	23.0	10	0.913	11.40

Table III—(Antistaphylococcal Activity, Y) = $aX^2 + bX + c$ ($X = R_m$)

Compound	a	b	c	n	r	s
Hexadecanoic acid	−18.6	87.4	30.4	10	0.845	9.70
cis-9-Hexadecenoic acid	8.1	76.6	12.2	10	0.893	12.40
Octadecanoic acid	−100.0	91.3	49.2	10	0.705	14.90
cis-9-Octadecenoic acid	−8.7	79.4	25.2	10	0.877	10.10
cis-9,*cis*-12-Octadecadienoic acid	8.1	68.9	37.4	10	0.624	11.00
cis-9,*cis*-12,*cis*-15-Octadecatrienoic acid	21.1	72.0	33.0	10	0.758	9.52
Eicosanoic acid	−17.3	88.4	27.5	10	0.861	10.70
Ethyl hexadecanoate	−4.2	109.0	19.6	10	0.885	11.00
Ethyl octadecanoate	−112.0	193.0	0.4	10	0.884	11.00
Ethyl *cis*-9-octadecenoate	41.8	65.9	10.3	10	0.911	11.40
Ethyl *cis*-9,*cis*-12-octadecadienoate	49.9	46.8	4.2	10	0.918	11.50
Ethyl eicosanoate	194.0	84.9	2.4	10	0.882	14.00
1-Hexadecanol	29.2	91.5	15.9	10	0.904	10.60
1-Octadecanol	30.2	84.0	7.7	10	0.917	10.90
cis-9-Octadecenol	14.0	88.3	14.5	10	0.900	10.70
White paraffin oil	12.9	86.4	23.0	10	0.838	12.20

Table IV—(Antistaphylococcal Activity, Y) = $aX^3 + bX^2 + cX + d$ ($X = R_m$)

Compound	a	b	c	d	n	r	s
Hexadecanoic acid	225.0	−159.0	81.7	33.8	10	0.866	9.59
cis-9-Hexadecenoic acid	599.0	−671.0	266.0	2.7	10	0.883	11.20
Octadecanoic acid	515.0	−194.0	34.7	47.8	10	0.851	14.70
cis-9-Octadecenoic acid	235.0	−212.0	99.5	99.5	10	0.871	9.44
cis-9,*cis*-12-Octadecadienoic acid	310.0	−56.4	19.1	40.2	10	0.926	10.30
cis-9,*cis*-12,*cis*-15-Octadecatrienoic acid	22.7	14.0	68.7	33.3	10	0.892	10.20
Eicosanoic acid	397.0	−290.0	96.6	32.6	10	0.869	9.76
Ethyl hexadecanoate	562.0	−382.0	148.0	22.1	10	0.871	11.30
Ethyl octadecanoate	973.0	−1080.0	450.0	−17.3	10	0.858	10.80
Ethyl *cis*-9-octadecenoate	195.0	−165.0	119.0	8.7	10	0.891	12.20
Ethyl *cis*-9-,*cis*-12-octadecadienate	91.7	−81.7	100.0	−1.1	10	0.900	12.40
Ethyl eicosanoate	0.0	194.0	84.9	2.4	10	0.882	14.00
1-Hexadecanol	340.0	−232.0	129.0	17.1	10	0.883	11.20
1-Octadecanol	292.0	−280.0	169.0	3.0	10	0.894	11.50
cis-9-Octadecenol	132.0	−102.0	108.0	15.0	10	0.874	11.50
White paraffin oil	−258.0	166.0	87.2	19.1	10	0.825	12.90

correlations in regression equations. The correlation coefficients and standard deviations of the regression equations also were determined.

RESULTS AND DISCUSSION

Tables II, III, and IV summarize the first-, second-, and third-order regression equations, respectively [where Y is biological response (antistaphylococcal activity), X is the R_m value, n is the number of points used (*i.e.*, number of compounds), r is the correlation coefficient, and s is the standard deviation of the regression equations]. First-order equations consistently resulted in the best correlations between R_m values and antistaphylococcal activity.

Comparison among the correlations obtained with saturated C_{18} fatty acids and C_{18} unsaturated acids indicated that improvement could be accomplished by using an unsaturated stationary phase, but no trend appeared evident between the amount of unsaturation and improvement.

The alcohols, ethyl eicosanoate, *cis*-9-octadecenol, 1-octadecanol, and 1-hexadecanol, gave essentially equivalent correlations, all being better than white paraffin oil. Comparison of the alcohols and their corresponding fatty acids, although limited, suggested slight superiority of the fatty acids.

Correlations of the esters, ethyl *cis*-9,*cis*-12-octadecadienoate, ethyl *cis*-9-octadecenoate, and ethyl hexadecanoate, were improved over white paraffin oil, but the correlation of ethyl eicosanoate was not. Among the esters, the best correlation was obtained with ethyl hexadecanoate. Comparison of the correlation of C_{18} esters with each other showed that all were approximately equal, although there was a slight decrease in correlation with increased unsaturation. The results also suggested that the esters did not give as great an improvement in the correlation between biological response and R_m values as did both the fatty acids and alcohols.

Of the 15 stationary phase materials investigated, 12 resulted in an improvement of the correlation between R_m and biological response over

that previously obtained with white paraffin oil. No definite relationships appeared evident when comparisons were made of chain length, amount of unsaturation, and functional group. The greatest improvement in the correlation between biological response and R_m for all stationary phases investigated was obtained with either *cis*-9,*cis*-12,*cis*-15-octadecatrienoic acid (linoleic acid) or hexadecanoic acid (palmitic acid).

Although no definite trend resulted from comparison between the stationary phase material and improvement of correlation, the following general relationship was found between functional group and improvement of correlation: fatty acids = alcohols > ethyl esters > white paraffin oil.

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ACKNOWLEDGMENTS

Presented in part at the Seventh Central Regional Meeting of the American Chemical Society, Morgantown, W.Va., May 1975, Abstract No. 60.

Abstracted in part from a dissertation submitted by K. E. Shores to the Graduate School, Xavier University, in partial fulfillment of the Master of Science degree requirements.

Supported in part by a grant from Stanley Drug Products, Inc., Division of Sperti Drug Products, Inc.

The authors thank Dr. Harvey A. Dube and Dr. Robert G. Johnson of the Chemistry Department, Xavier University, for their valuable suggestions.

Amino Acid Analogs IV: 4-Fluoroisoleucine

HERMAN GERSHON *x, LARRY SHANKS *, and DONALD D. CLARKE ‡

Received April 28, 1977, from the **Boyce Thompson Institute for Plant Research, Yonkers, NY 10701*, and the ‡*Department of Chemistry, Fordham University, Bronx, NY 10458.* Accepted for publication August 23, 1977.

Abstract □ 4-Fluoroisoleucine was produced by ammonolysis of 2-bromo-4-fluoro-3-methylpentanoic acid, which resulted from the bromofluorination of 4-methyl-2-pentenoic acid. It did not inhibit *Plasmodium berghei* in mice at 640 mg/kg and was not toxic to the animals. The fluoroamino acid inhibited *Aspergillus niger, Trichoderma viride, Myrothecium verrucaria, Trichophyton mentagrophytes,* and *Mucor mucedo* in Czapek solution agar at a concentration between 10^4 and 10^3 μg/ml. Growth of *Escherichia coli* was inhibited 25% at 900 μg/ml in a defined medium.

Keyphrases □ 4-Fluoroisoleucine—synthesized, evaluated for antimicrobial activity □ Antimicrobial activity—4-fluoroisoleucine evaluated □ Amino acid analogs—4-fluoroisoleucine synthesized and evaluated for antimicrobial activity

The preparation of straight chain 3-fluoroamino acids with three to seven carbon atoms as well as 3-fluorovaline was reported (1). The general approach employed started with the bromofluorination of the corresponding 2-alkenoic acid followed by ammonolysis.

DISCUSSION

In attempting to prepare 3-fluoroleucine, 4-methyl-2-pentenoic acid (2) was dissolved in liquid hydrofluoric acid with the subsequent addition of *N*-bromoacetamide. The expected product was not obtained. The resulting mixture was composed of two major products: 2-bromo-4-fluoro-3-methylpentanoic acid and 2-bromo-4,4-dimethyl-4-butyrolactone. The 2-bromo-4-fluoro-3-methylpentanoic acid was converted to 4-fluoroisoleucine by ammonlysis in liquid ammonia.

Identification of these compounds was made by elemental analysis and NMR spectroscopy (see *Experimental*). IR spectra of the three compounds were obtained (Fig. 1).

The neutral product obtained from the addition of the bromo and fluoro elements to 4-methyl-2-pentenoic acid contained no fluorine and showed two singlets in the NMR spectrum corresponding to three protons each at δ 1.48 and 1.63 ppm. This result indicated the fragment $(CH_3)_2C(-X)C$, in which X is an electronegative group that shifts the methyl groups from their original position near δ 1.10 ppm. The classic *ABX* spectrum located between δ 2.47–2.79 (*AB* portion) and 4.7 (*X* portion) ppm, with J_{AB} = 14.5 Hz, indicated geminal coupling of the protons on a saturated carbon atom. This result suggested the presence of the fragment CH_2CHBr. The IR spectrum of the compound (Fig. 1A) showed a strong peak at 1780 cm^{-1}. The spectral data were in agreement with the structure of the compound being 2-bromo-4,4-dimethylbutyrolactone.

The structures of the bromofluoro and fluoroamino acids were deduced from the NMR and IR spectra as follows. That fluorine was attached to a carbon atom bearing a proton was evident from the 49-Hz coupling to the proton centered at δ 5.2 ppm. This coupling was expected for the anticipated product, 2-bromo-3-fluoro-4-methylpentanoic acid. Instead of a doublet near δ 1.10 ppm (J = 7 Hz) of an area corresponding to six protons, characteristic of the isopropyl group as in the starting acid, the product of bromofluorination of 4-methyl-2-pentenoic acid showed a doublet at δ 1.12 ppm (J = 7 Hz) and a doublet of doublets centered at δ 1.37 ppm (J_{HH} = 6 Hz; J_{HF} = 29 Hz).

A comparison of the 60- and 100-MHz spectra indicated that the 29-Hz separation was a coupling constant rather than a chemical shift. The NMR spectrum showed that a rearrangement involving the isopropyl group of the starting acid had taken place. The doublet of doublets is indicative of the fragment CH_3CHF and was reported previously in the product of the addition of the bromo and fluoro elements to crotonic acid (1). Further confirmation that fluorine was present in the molecule and was coupled to the methyl group at δ 1.37 ppm and to the protons at C-3