SYNTHESES OF ^{14}C-MUSTARD-LABELLED AND ^{14}C-LEUCINE-LABELLED ASALEY

D. Farquhar, K. Lu, and T. L. Loo
Department of Developmental Therapeutics,
The University of Texas System Cancer Center
M. D. Anderson Hospital and Tumor Institute
Houston, Texas 77030.

SUMMARY

The synthesis of Asaley labelled with ^{14}C in the mustard (Asaley-must-^{14}C) or the leucine (Asaley-leu-^{14}C) moiety is described. In the former, ethyl p-amino-N$^{\alpha}$-acetyl-DL-phenylalanyl-L-leucinate was reacted with ethylene oxide-(U)-^{14}C to give the bis(hydroxyethyl)amino analogue, which was converted to Asaley by treatment with thionyl chloride. Asaley labelled in the leucine moiety was prepared by condensation of N-acetylsarcolysin with ethyl leucinate-(U)-^{14}C in the presence of dicyclohexylcarbodiimide. The identity of the products was established by spectroscopic, chemical, and chromatographic techniques.

Key words: Asaley, Sarcolysin, D,L-phenylalanine mustard, antitumor.

INTRODUCTION

Asaley (ethyl p-[bis{2-chloroethyl}amino]-N$^{\alpha}$-acetyl-DL-phenylalanyl-L-leucinate; NSC-167,780), an alkylating agent first synthesized in the Soviet Union, is a dipeptide derivative of N-acetylsarcolysin. Because of reported

0362-4803/80/0217-0159$01.00

Received November 22, 1978
Revised March 13, 1979

superior antitumor activity to sarcolysin (D,L-phenylalanine mustard; NSC-14210) in a number of experimental (1,2,3) and human (3) malignancies, Asaley is undergoing clinical evaluation in the United States(4). As an aid to biochemical pharmacologic studies in experimental animals and in man, we required Asaley labelled with ^{14}C in the nitrogen mustard (i.e., bis{2-chloroethyl} amino) moiety and in the leucine moiety. The syntheses of these compounds are described.

MATERIALS AND METHODS

Ethylene oxide-(U)-^{14}C (19.1 mCi/mmole), contained in a "breakseal" ampule fitted with a 14/20 male joint, was purchased from Schwartz/Mann, Orangeburg, New York. L-leucine-(U)-^{14}C (312 mCi/mmole), in 0.01 N HCl, was obtained from New England Nuclear, Boston, Massachusetts. Sarcolysin (NSC-14210) and Asaley (NSC-167,780) were generously provided by the Drug Development Branch, Drug Research and Development, Division of Cancer Treatment, National Cancer Institute. Column chromatography was carried out on Woelm neutral aluminum oxide, activity grade I (Alupharm Chemicals, New Orleans). Preparative and thin layer chromatography were performed on glass plates coated with silica gel 60 (F-254) (E. Merck, Darmstadt, Germany); the chromatograms were viewed under short wavelength (254 nm) ultraviolet light. To detect alkylating activity, the plates were sprayed with a 1% solution of 4-(p-nitrobenzyl)-pyridine (NBP) in acetone, then heated at 100° for 5 minutes, and sprayed with a 2% solution of sodium hydroxide in ethanol; alkylating compounds appeared as blue spots against a white background. Aromatic amines were detected by spraying the chromatograms with a 1% solution of p-dimethylaminobenzaldehyde (DMAB) in ethanol-12N hydrochloric acid (95:5); yellow spots against a white background were produced. Radioactivity was determined with a Packard Model 3375 Tricarb liquid scintillation spectrometer using "PCS" (Amersham/Searle)

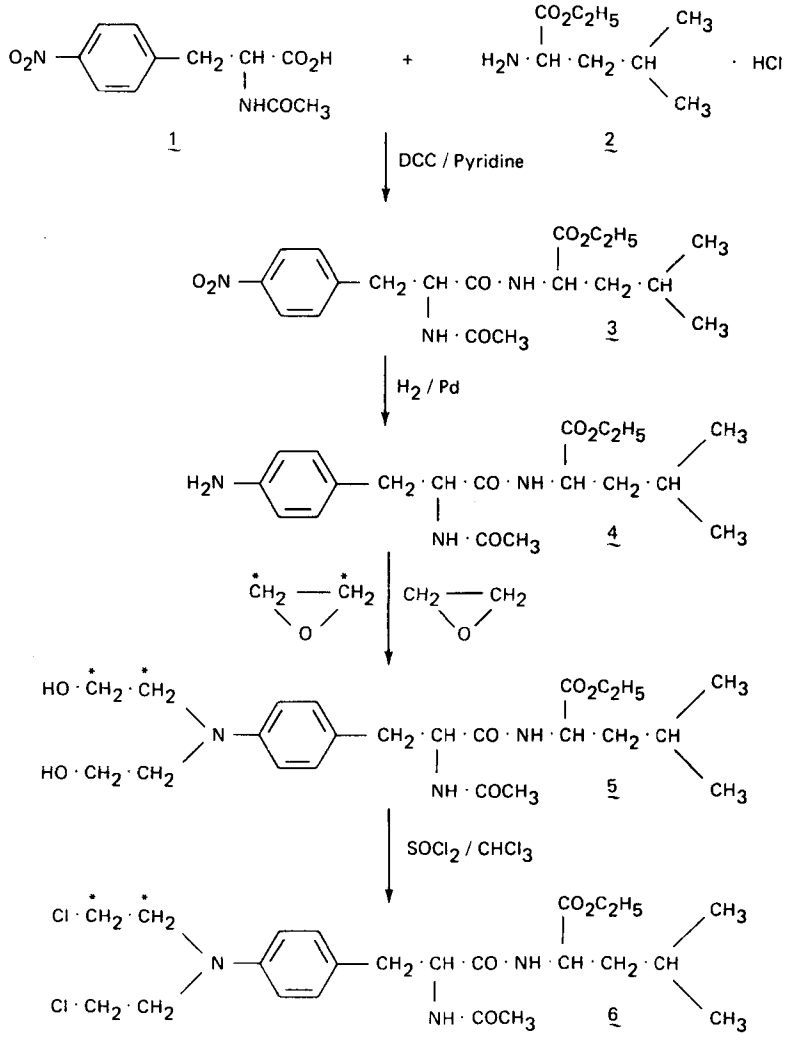
Associates T-60A spectrophotometer using tetramethylsilane as an internal standard. Mass spectra were obtained on a Finnigan Model 3000 quadrapole mass spectrophotometer. Optical rotations were measured in 1-dm tubes with a Schmidt-Haensch polarimeter. Melting points were determined on a Fischer-Johns melting point apparatus and are uncorrected. Elemental analyses were performed by Galbraith Laboratories, Knoxville, Tennessee; results were within ±0.4% of theoretical values.

RESULTS AND DISCUSSION

Asaley labelled with ^{14}C in the nitrogen mustard group was synthesized as indicated in Scheme 1. Ethyl p-nitro-N$^{\alpha}$-acetyl-DL-phenylalanyl-L-leucinate (3) was prepared by dicyclohexylcarbodiimide-induced condensation of N-acetyl-p-nitrophenylalanine (1) and ethyl L-leucinate hydrochloride (2), in pyridine. Reduction of 3 with hydrogen over 10% palladium-on-charcoal afforded the free amine, 4. Reaction of 4 with ethylene oxide-(U)-^{14}C in glacial acetic acid, at room temperature, yielded the bis(hydroxyethyl)amino analogue, 5. To ensure maximum utilization of the radiolabel, the ethylene oxide-(U)-^{14}C was initially reacted with a large (>30-fold) molar excess of the amine. Unlabelled ethylene oxide was then added to drive the reaction to completion. Since the reaction proceeds through the intermediacy of a monohydroxyethylamino adduct, the majority of the radiolabel in 5 is assumed to be present in one, rather than both, of the hydroxyethyl groups. The utilization of ethylene oxide-^{14}C, based on the specific activity of the product, was 86%. Conversion of 5 to Asaley (6) was accomplished by treatment with thionyl chloride in anhydrous chloroform. Although a significant amount of decomposition product was formed during the reaction, Asaley was readily isolated by chromatography of the crude reaction mixture on thick layers of silica. A trace amount of the monochloroethyl analogue, together with some unreacted starting material, was also recovered.

SCHEME 1

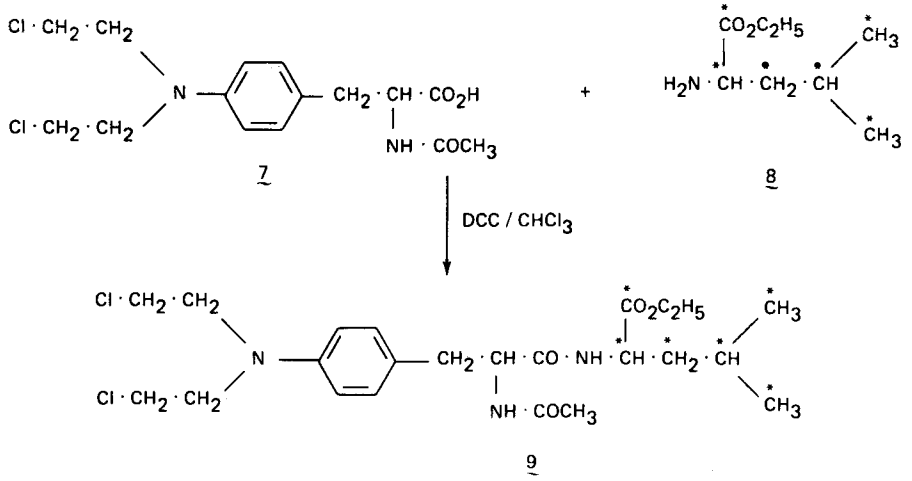
SYNTHESIS OF ASALEY – Must-^{14}C



Asaley labelled uniformly with ^{14}C in the leucine moiety was synthesized as outlined in Scheme 2.

SCHEME 2

SYNTHESIS OF ASALEY - Leu-^{14}C



Ethyl L-leucinate-(U)-^{14}C (8) was prepared from L-leucine-(U)-^{14}C via the intermediacy of the corresponding amino acid ester hydrochloride. Reaction of 8 with N-acetylsarcolysin (7) in the presence of dicyclohexylcarbodiimide afforded Asaley-leu-^{14}C (9) in 60% yield.

Unlabelled Asaley, prepared by the same synthetic sequence as 6 or 9, was identical, by spectral (NMR, MS, OR), chemical, physicochemical, and chromatographic comparison, to an authentic sample of the drug.

The position of the label in 6 and 9 was confirmed by chemical hydrolysis with 6 N HCl; quantitative radiochemical yields of L-sarcolysin-^{14}C and L-leucine-^{14}C, respectively, were recovered (Table 1).

TABLE 1

Chromatography of Asaley-must-^{14}C, Asaley-leu-^{14}C, and hydrolysis products on Whatman No 1 paper [Eluent: n-butanol - 95% ethanol - propionic acid - water (10:5:2:5)]

Compound or product	R_f	Reaction with		% Recovery
		NBP	Ninhydrin	of radioactivity
Asaley-must-^{14}C	0.95	+	-	100
Asaley-leu-^{14}C	0.95	+	-	100
Asaley-must-^{14}C hydrolysate	0.81	+	+	100
	0.67	-	+	0
Asaley-leu-^{14}C hydrolysate	0.81	+	+	0
	0.67	-	+	99
Sarcolysin	0.81	+	+	N/A
L-Leucine	0.67	-	+	N/A

N/A = Not Applicable

EXPERIMENTAL

Ethyl p-nitro-N$^{\alpha}$-acetyl-DL-phenylalanyl-L-leucinate (3).

Dicyclohexylcarbodiimide (12.3 g, 59.6 mmole) was added to a solution of N-acetyl-p-nitro-DL-phenylalanine[5] (1, 14.0 g, 59.5 mmole) and ethyl L-leucinate hydrochloride[6] (2, 11.7 g, 59.5 mmole) in dry pyridine (1000 mL), and the mixture was stirred at room temperature for six days. The precipitate of N,N-dicyclohexylurea was filtered off, and the pyridine was evaporated in vacuo. Chloroform (300 mL) was added to the residual oil, and the solution was

filtered to remove additional N,N-dicyclohexylurea. The filtrate was washed successively with 3 x 100 mL portions of saturated sodium bicarbonate solution, 1 x 100 mL portion of water, and was dried over anhydrous sodium sulfate. The chloroform was evaporated *in vacuo*, and the residue was recrystallized from benzene-heptane to give 3 as white platelets. Yield-14.4 g (61%); mp 156-158°; M^+(m/e)=393; NMR ($CDCl_3$)δ8.12 (d, 2H, C_3- and C_5-H), 7.38 (d, 2H, C_2- and C_6-H), 5.00 (m, 1H, $\underline{CH}NHCOCH_3$), 4.42 (m, 1H, $\underline{CH}NHCO_2C_2H_5$), 4.18, 4.15 (2xq, 2H, $O\underline{CH_2}CH_3$), 3.17 (d, 2H, $Ar\underline{CH_2}$), 1.95 (s, 3H, $COCH_3$), 1.0-1.8 (m, 2H, $\underline{CH_2}CH(CH_3)_2$), 1.27, 1.24 (2xt, 3H, $OCH_2\underline{CH_3}$), 0.7-1.1 (m, 7H, $CH(CH_3)_2$). Anal: Calcd for $C_{19}H_{27}N_3O_6$: C, 58.00; H, 6.92; N, 10.68. Found: C, 58.12; H, 7.04; N, 10.80.

Ethyl p-amino-N$^{\alpha}$-acetyl-DL-phenylalanyl-L-leucinate (4)

A solution of 3 (8.7 g, 22.1 mmole) in absolute ethanol (300 mL) was hydrogenated at 40 psi over 10% palladium-on-charcoal (500 mg) for 90 minutes at room temperature. The mixture was filtered through a pad of diatomacious earth (Celite), and the filtrate was concentrated *in vacuo*. The gummy residue solidified on trituration with benzene-heptane (1:1). Recrystallization of the crude product from ethyl acetate afforded 4 as white needles-Yield: 6.1 g (76%); mp 160-162°; M^+(m/e)=363; NMR ($CDCl_3$)δ 7.02 (d, 2H, C_2- and C_6-H), 6.60 (d, 2H, C_3- and C_5-H), 4.67 (m, 1H, $\underline{CH}NHCOCH_3$), 4.40 (m, 1H, $\underline{CH}NHCO_2C_2H_5$), 4.17 (q, 2H, $O\underline{CH_2}CH_3$), 3.62 (broad band, 2H, NH_2), 3.30 (d, 2H, $Ar\underline{CH_2}$), 1.93 (s, 3H, $COCH_3$), 1.0-1.7 (m, 2H, $\underline{CH_2}CH(CH_3)_2$), 1.23 (t, 3H, $OCH_2\underline{CH_3}$), 0.65-1.0 (m, 7H, $\underline{CH(CH_3)_2}$). Anal: Calcd for $C_{19}H_{29}N_3O_4$: C, 62.79; H, 8.04; N, 11.56. Found: C, 62.83; H, 8.32; N, 11.56.

Ethyl p-[bis(2-hydroxyethyl-^{14}C)amino]-N$^{\alpha}$-acetyl-DL-phenylalanyl-L-leucinate (5)

A "break-seal" tube containing ethylene oxide (1 mCi; 19.1 mCi/mmole) was partially immersed in a dry-ice/acetone bath at -78° for 30 minutes. The seal was then broken, and the tube was quickly fitted on top of a 25 ml

single-neck (14/20) flask containing a solution of 4 (0.68 g, 1.87 mmole) in glacial acetic acid (5.0 ml). To ensure a tight fit, the joints were lightly smeared with high vacuum grease, and the assembly was clamped together. The solution was stirred for 42 hours at room temperature. The flask was then immersed in an ice-salt bath at -10° for 30 minutes, and liquid ethylene oxide (1.0 ml) was added. The apparatus was quickly resealed, and the reaction mixture was stirred for a further 24 hours at room temperature. After cooling to -10°, the flask was opened, and the contents was allowed to warm to room temperature. The mixture was evaporated in vacuo, and the residual viscous oil was taken up in chloroform (10 mL). The solution was washed successively with 2 x 5 mL portions of saturated sodium bicarbonate solution, 1 x 5 mL portion of water, and was dried over anhydrous sodium sulfate. The solvent was evaporated, and the residue was dissolved in chloroform (5 mL) and transferred to a column of aluminum oxide (100 g) made up in the same solvent. The mixture was eluted with chloroform-methanol (98:2); fractions of 5 mL each were collected. The rate of elution was monitored by subjecting 10 µL aliquots of alternate fractions to tlc on silica in chloroform-methanol (9:1). The minor, pale-yellow forerunner band (R_f=0.44, DMAB positive), tentatively identified from its mass spectrum [M^+(m/e)=407] as the monohydroxyethylamino analogue of 6, was not further investigated. The colorless second band (R_f = 0.31, DMAB negative) yielded a viscous oil that solidified on trituration with ether; it was recrystallized from methanol/ether to give 6 as white needles - Yield: 0.57 g (68%); spec. act. = 0.4 mCi/mmole. The utilization of ethylene oxide-^{14}C, based on the specific activity of 5, was 86%. Chemical and spectral analyses were conducted on a sample of the unlabelled compound prepared by the same synthetic route - m.p = 122-123° (ethanol-ether); M^+(m/e) = 451; NMR ($CDCl_3$)δ 6.93 (d, 2H, C_2- and C_6-H), 6.57 (d, 2H, C_3- and C_5-H), 4.0-5.0 (m, 4H, $\underline{CH}NHCO_2C_2H_5$, $\underline{CH}NHCOCH_3$, and $(\underline{HO}CH_2CH_2)_2$), 4.13 (q, 2H, $O\underline{CH_2}CH_3$), 2.92 (d, 2H, $Ar\underline{CH_2}$), 1.92 (s, 3H, $COCH_3$),

1.0-1.8 (m, 2H, $\underline{CH_2}CH(CH_3)_2$), 0.5-1.0 (m, 7H, $\underline{CH(CH_3)_2}$). Anal: Calcd for $C_{23}H_{37}N_3O_6$: C, 61.17; H, 8.26; N, 9.31. Found: C, 61.22; H, 8.47; N, 9.32.

Ethyl p-[bis(2-chloroethyl-^{14}C)amino]-N$^{\alpha}$-acetyl-DL-phenylalanyl-L-leucinate (Asaley-must-^{14}C, 7).

Thionyl chloride (0.7 mL) was added rapidly to a stirred solution of 6 (0.50 g, 1.11 mmoles) in anhydrous chloroform (25 mL). The mixture immediately turned cloudy, and a colorless mobile oil separated. The flask was immersed in a water bath at 45°, and the reaction mixture was stirred vigorously for 90 minutes under a dry nitrogen atmosphere. The dark brown solution was evaporated under reduced pressure at < 40°. The gummy oil was taken up in chloroform (25 mL), and the solution was re-evaporated. Trituration of the residue with anhydrous ether yielded a light brown amorphous solid (494 mg). Tlc of a portion of the product on silica, using chloroform-methanol (9:1), revealed three components: (1) a major, NBP-positive band at R_f = 0.58, identified as Asaley by chromatographic comparison with an authentic sample, (2) a minor, NBP-positive band at R_f = 0.39, identified from its mass spectrum [M^+(m/e)=469, 471] as the monohydroxyethyl analogue of Asaley, and (3) a minor, NBP-negative band at R_f = 0.29, identified as unreacted 6. The crude product was dissolved in chloroform (10 mL), and the solution was applied to five thick-layer plates of silica. The chromatograms were developed with chloroform-methanol (9:1). Bands corresponding to Asaley were scraped-off, combined, crushed to a uniform powder in a mortar and pestle, and transferred to a glass column (30 x 2.2 cm) with the aid of several washings of chloroform. Asaley was eluted with chloroform-methanol (8:2). Evaporation of the solvent yielded a pale yellow viscous residue that solidified on drying under vacuum. The product was washed with ether, dried, and stored under nitrogen over phosphorus pentoxide at -20°. Yield: 292 mg (54%); spec. act. = 0.46 mCi/mmole. Tlc of a portion of the product on silica in chloroform-methanol (9:1) showed a single, NBP-positive spot at R_f = 0.58. To determine the radiochemical homogeneity of the

product, 1 cm bands of the chromatogram were scraped directly into liquid scintillation counting vials; water (1 mL) was added, followed, after 15 minutes, by counting fluid (15 mL). The "cocktails" were shaken vigorously for 1 minute and were then counted. Over 99.9% of the radioactivity was located in the region of the chromatogram corresponding to Asaley.

Unlabelled Asaley was similarly prepared from unlabelled 5. A sample for analyses was recrystallized from ethanol/ether: pale yellow needles, mp 105-110° [Lit[3] 106-110°]; $[\alpha]_D^{25}$ = -5° [c = 2.0, ethanol]; M^+(m/e) = 487, 489, 491; NMR ($CDCl_3$)δ 7.10 (d, 2H, C_2- and C_6-H), 6.65 (d, 2H, C_3- and C_5-H), 4.3-5.0 (m, 2H, $ArCH_2$), 1.95 (s, 3H, $COCH_3$), 1.3-1.8 (m, 2H, $\underline{CH_2}CH(CH_3)_2$), 1.28, 1.26 (2xt, 3H, $OCH_2\underline{CH_3}$), 0.7-1.1 (m, 7H, $\underline{CH(CH_3)_2}$). Anal: Calcd for $C_{23}H_{35}N_3O_4Cl_2$ C, 56.55; H, 7.22; N, 8.60. Found: C, 56.69; H, 7.46; N, 8.74.

Ethyl leucinate-(U)-^{14}C hydrochloride (8)

A solution of L-leucine-(U)-^{14}C hydrochloride (1 mCi, 312 mCi/mmole), in 0.01 N HCl (10 mL), contained in a 250 ml round-bottomed flask, was evaporated to dryness in vacuo. The residue was dissolved in anhydrous ethanol (75 mL) and the solution was again evaporated. L-leucine (0.36 g; 2.75 mmoles) was added, followed by anhydrous ethanol (75 mL), and the suspension was saturated, while stirring, with dry hydrogen chloride. The clear solution obtained was refluxed for 1 hour while protected from atmosphere moisture. The solution was left at room temperature for another hour and was then evaporated to dryness in vacuo. The residue was dissolved in ethanol (5 mL); ether (75 mL) was added, and the solution was stored overnight at 0-5°. The precipitate was filtered, washed with ether, and dried in vacuo over sodium hydroxide pellets. The product was recrystallized from ethanol-ether to give 8 as glistening white plates - Yield: 0.47 g (88%), spec. act. = 0.36 mCi/mmole.

Ethyl leucinate-(U)-^{14}C (9)

Ether (10 mL) was added to a solution of 8 (0.44 g, 2.25 mmoles) in water (10 mL) and the mixture was cooled to 0° on an ice-bath. A 25% aqueous solution

(0.5 mL) of sodium hydroxide was added, dropwise, with rapid stirring. The ether layer was separated, and the aqueous layer was washed with 3 x 10 mL portions of ether. The ethereal extracts were combined, dried over anhydrous sodium sulfate, filtered, and evaporated at 25° under reduced pressure (30 Torr); 9 was obtained as a colorless mobile oil - Yield: 0.32 g (89%).

Ethyl p-[bis(2-chloroethyl)amino]-N$^{\alpha}$-acetyl-DL-phenylalanyl-L-leucinate-(U)-^{14}C (Asaley-leu-^{14}C, 9)

A mixture of 9 (0.30 g, 1.89 mmole), N-acetylsarcolysin (0.66 g, 1.90 mmole), and dicyclohexylcarbodiimide (0.4 g, 1.94 mmole) in dry chloroform (10 mL) was stirred for 48 hours at room temperature. The precipitate of dicyclohexylurea was filtered off, and the solution was evaporated *in vacuo*. The yellow viscous residue was purified on five preparative layer plates, as described for 6 - Yield: 0.55 g (60%), spec. act. = 0.36 mCi/mmole. The radiochemical purity of the compound, determined as described for 6, was 99.9%.

The unlabelled compound was similarly prepared: pale yellow needles from ethanol-ether, mp 103-110° [Lit[3] 106-110°]; $[\alpha]_D^{25}$ = -5° (c = 2.0, ethanol); M^+(m/e) = 487, 489, 491; NMR ($CDCl_3$), identical to unlabelled 7.

Hydrolysis of Asaley-must-^{14}C and Asaley-leu-^{14}C

A solution of 6 or 9 (1 mg) in 6 N hydrochloric acid (2 mL) was heated under reflux for 24 hours. Aliquots of the hydrolysates were spotted on Whatman No 1 paper. Unlabelled sarcolysin and leucine were used as standards. The chromatograms were developed in n-butanol - 95% ethanol - propionic acid - water (10:5:2:5; v/v). Alkylating substances were detected with the NBP reagent, and amino acids were located with ninhydrin. To determine the position of radioactivity, the chromatograms were cut into 1 cm strips. Each strip was placed in a counting vial; water (1 mL) was added, followed, after 1 hour, by liquid scintillation fluid (15 mL). The cocktails were shaken for 1 minute and then counted.

REFERENCES

1. Zaytseya, L.A., Borodkina, R.P., Lopatin, P.V., and Syrkin, A.B. Pharmakol. Toksikol, 4:460 (1971).
2. Lationov, L.F., Bukhasova, I.K., and Shanayeva, Ye.M. Vopr. Onkol II: 78 (1965).
3. Regulations for the Production of Asaley. An NIH Library Translation (NIH 73-132-C) of the Product Information Brochure from the USSR.
4. Bodey, G.P., Gottlieb, J.A., Burgess, M.A., and Alexanian, R. Med. Pediatr. Oncol. 3:365 (1977).
5. Bergel, F., Burnop, V.C.E., and Stock, J.A. J. Chem. Soc. 1223 (1965).
6. a) Rohman, F., Ber., 30:1978 (1897)
   b) Greenstein, J.P., and Winitz, M. The Chemistry of the Amino Acids, Vol. 2, John Wiley and Sons, New York (1961), p. 927.
7. Shkodinskaya, M.N., Vasil'eva, M.N. Vasina, O.S., and Berlin, A. Ya. Zhur. Obschel Khim. 29:3776 (1959), through Chem. Abs. 54:22474 (1960).

ACKNOWLEDGEMENT

This work was supported by Contract NOI-CM-87185 with the Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, U.S. Public Health Service.