Substrates for Cytochemical Demonstration of Enzyme Activity. II. Some Dihalo-3-indolyl Phosphates and Sulfates[1]

JEROME P. HORWITZ, JONATHAN CHUA, MICHAEL NOEL, JOSEPH T. DONATTI, AND JOSEF FREISLER

The Rollin H. Stevens Memorial Laboratory of the Detroit Institute of Cancer Research, Detroit, Michigan 48201

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A recent communication[2] from this laboratory described the syntheses of several dihalo-3-indolyl-β-D-glycopyranosides for the histochemical demonstration of corresponding glycosidases in mammalian tissue. The utility[3] of these substrates derives from the rapid deposition of an insoluble, microcrystalline tetrahaloindigo at the sites of activity through oxidation of an enzymically released intermediate dihaloindoxyl. This same chromogenic reaction sequence has been applied to the localization of alkaline phosphatase through the use of both sodium 3-indolyl phosphate[4] and calcium 5-bromo-3-indolyl phosphate.[5] The fact that improved staining results[3,6] are obtained with indoxyl derivatives that carry 5,6- and in particular 5,4-bromochloro substituents prompted the preparation of the corresponding dihalo-3-indolyl phosphates. Moreover, indigogenic staining has been extended to the localization of arylsufatase(s) through syntheses of 5,6- and 5,4-bromochloro-3-indolyl sulfates.

Experimental Section[7]

p-Toluidinium 5-Bromo-6-chloro-3-indolyl Phosphate.—To a suspension of 0.5 g (1.69 mmoles) of 1-acetyl-5-bromo-6-chloroindol-3-ol[2] in 5 ml of dry pyridine, cooled to −20°, was added 0.2 ml (2.18 mmoles) of $POCl_3$ and the reaction mixture was stirred magnetically at 0° overnight (18 hr) with care to exclude moisture. The dark suspension was then stirred at room temperature for an additional 5 hr to achieve complete homogeneity. The amber solution was evaporated to dryness and the $-POCl_2$ intermediate was hydrolyzed by the addition of ice (*ca.* 5 g). The aqueous mixture was adjusted to pH 9 with 10% KOH and the solution was evaporated to dryness. Traces of water were removed from the residue after three evaporations from 5-ml portions of absolute ethanol. The solid was dissolved in 5 ml of methanol containing 1 equiv of $KOCH_3$ and the solution was held at room temperature overnight. The reaction mixture was then neutralized with acetic acid and evaporated to dryness. The residue was dissolved in 5 ml of water, decolorized with Norit, and the filtrate was treated with a solution of 0.250 g (1.72 mmoles) of *p*-toluidine hydrochloride in 5 ml of water. The off-white solid that was deposited was collected, air dried, and then crystallized (Norit) from absolute ethanol; 0.38 g (51% yield), mp 198–200° dec.

Anal. Calcd for $C_{15}H_{15}BrClN_2O_4P$: C, 41.54; H, 3.49; N, 6.46; P, 7.14. Found: C, 41.34; H, 3.67; N, 6.80; P, 7.55.

p-Toluidinium 5-Bromo-4-chloro-3-indolyl Phosphate.—The extension of the above method to 1-acetyl-5-bromo-4-chloroindol-3-ol[8] required only that the period of stirring at room temperature be increased to 24 hr. From 0.5 g (1.69 mmoles) of the indoxyl derivative there was obtained 0.3 g (45% yield) of product which crystallized from absolute ethanol in the form of colorless short needles, mp 194–195° with immediate resolidification of the melt and ultimate decomposition at 230°.

Anal. Found: C, 41.49; H, 3.73; N, 6.66.

Potassium 5-Bromo-4-chloro-3-indolyl Sulfate.—To a solution of 0.25 ml (4.3 mmoles) of chlorosulfonic acid in 5 ml of pyridine cooled to 0°, was added, all at once, 1.0 g (3.3 mmoles) of 1-acetyl-5-bromo-6-chloroindol-3-ol. The mixture was stirred magnetically at 0° for 40 hr followed by an additional 4 hr at room temperature. The deep red solution was evaporated to dryness and the residue was treated with *ca.* 1 ml of ice water. The solution was again evaporated and the last traces of moisture were removed by several evaporations from ethanol. The oil was dissolved in a small volume of methanol, and the pH of the solution was adjusted to 9–10 with a saturated solution of methanolic KOH. After *ca.* 10 min, the reaction mixture was neutralized with glacial acetic acid and evaporated to dryness. The product crystallized from a small volume (*ca.* 2 ml) of water in the form of colorless plates, after prior treatment with Norit; 0.715 g (60% yield), mp 200–202° dec after drying at 100° (10^{-3} mm).

Anal. Calcd for $C_8H_4BrClKNO_4S$: C, 26.35; H, 1.11; N, 3.84. Found: C, 25.94; H, 1.42; N, 3.91.

The product was characterized further as a *p*-toluidine salt which was obtained from the dissolution of equimolar quantities of the potassium salt and *p*-toluidine hydrochloride in a minimum of water at 60°. The product crystallized from water as colorless plates, mp 109–113° dec.

Anal. Calcd for $C_{15}H_{14}BrClN_2O_4S$: C, 41.54; H, 3.25; N, 6.46; S, 7.39. Found: C, 41.68; H, 3.21; N, 6.66; S, 7.62.

Potassium 5-Bromo-6-chloro-3-indolyl Sulfate.—The application of the procedure described above to 1-acetyl-5-bromo-4-chloroindol-3-ol provided the product in 26% yield, mp 170–175° dec.

Anal. Found: C, 26.16; H, 1.24; N, 4.04.

The conversion of the potassium salt to the corresponding toluidine derivative was accomplished as described above, mp 170–175° dec.

Anal. Found: C, 41.33; H, 3.40; N, 6.45.

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N-Substituted DL-Aspartic Acids and β-Methyl Esters

MARVIN J. KARTEN,[1] ERNEST MAGNIEN, ALLAN SCHWINN, AND SEYMOUR L. SHAPIRO

U. S. Vitamin and Pharmaceutical Corporation, Yonkers, New York 10701

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In continuation of our investigation of compounds which are effective in lowering blood cholesterol levels,[2] a number of additional N-substituted aspartic acid derivatives were prepared.

Experimental Section[3]

N-Substituted DL-Aspartic Acids β-Methyl Esters.—A modification of the method of Zilkha and Bachi[4] was employed. Maleic anhydride (0.22 mole) was refluxed in 60 ml of methanol for 0.5 hr. After cooling, 40 ml of pyridine, followed by 0.2 mole of the amine was added, and the solution was heated at 100–110° (oil bath) for 0.5 hr while 30 ml of methanol was removed. After cooling, 50 ml of ether was added to the yellow reaction mixture, and the resulting solid was filtered, washed with several 50-ml portions of ether, and recrystallized. The new esters, thus prepared, are listed in Table I.

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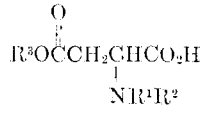
TABLE I

N-SUBSTITUTED DL-ASPARTIC ACIDS AND β-METHYL ESTERS



No.	R^1	R^2	R^3	Mp, °C	Recrystn solvent[a]	Yield, %	Formula	Calcd, % C	Calcd, % H	Calcd, % N	Found, % C	Found, % H	Found, % N
1	2-$ClC_6H_4CH_2$	H	H	216–218	A	90	$C_{11}H_{12}ClNO_4$	51.27	4.70	5.44	51.23	4.88	5.31
2	$C_6H_5CH_2CH_2$	H	H	189–190	A	90	$C_{12}H_{15}NO_4$	60.75	6.37	5.90	60.72	6.51	5.81
3	2-$ClC_6H_4CH_2CH_2$	H	H	200–201	B	33	$C_{12}H_{14}ClNO_4$	53.05	5.19	5.16	53.14	5.19	5.26
4	3,4-$(CH_3O)_2C_6H_3CH_2CH_2$	H	H	179–180	C	56	$C_{14}H_{19}NO_6$	56.56	6.44	4.71	56.36	6.48	4.92
5	$C_6H_5CH_2CH(CH_3)$	H	H	205–207	A	45	$C_{13}H_{17}NO_4$	62.14	6.82	5.57	62.08	6.77	5.98
6	2-$C_5H_4NCH_2$[b]	H	H	205–208	A	34	$C_{10}H_{12}N_2O_4$	53.57	5.39	12.50	53.32	5.40	12.80
7	3-$C_5H_4NCH_2$[b]	H	H	232–233	A	50	$C_{10}H_{12}N_2O_4$	53.57	5.39	12.50	53.29	5.59	12.90
8	C_6H_{11}[c]	C_6H_{11}[c]	H	242–243	D	59	$C_{18}H_{27}NO_4$	64.62	9.15	4.71	64.82	8.97	4.92
9	$-(CH_2)_4-$		H	176–178	E	47	$C_8H_{13}NO_4$	51.33	7.00	7.48	51.23	7.06	7.12
10	$-(CH_2)_5-$		H	183–185	A	90	$C_9H_{15}NO_4$	53.72	7.51	6.96	53.76	7.78	6.78
11	$-CH_2CH_2OCH_2CH_2-$		H	181–183	D	38	$C_8H_{13}NO_5$	47.29	6.45	6.89	47.29	6.51	6.64
12	2-$ClC_6H_4CH_2$	H	CH_3	185–186	E	42	$C_{12}H_{14}ClNO_4$	53.04	5.19	5.16	52.73	5.32	5.22
13	3-$ClC_6H_4CH_2$	H	CH_3	217–218	F	76	$C_{12}H_{14}ClNO_4$	53.04	5.19	5.16	52.85	5.22	5.30
14	4-$ClC_6H_4CH_2$	H	CH_3	223–224	A	44	$C_{12}H_{14}ClNO_4$	53.04	5.19	5.16	53.16	5.25	1.96
15	$C_6H_5CH(CH_3)$	H	CH_3	221–222	A	47	$C_{13}H_{17}NO_4$	62.14	6.82	5.58	62.16	6.50	5.47
16	$C_6H_5CH_2CH_2$	H	CH_3	238–239	E	62	$C_{13}H_{17}NO_4$	62.14	6.82	5.58	62.06	6.80	5.41
17	2-$ClC_6H_4CH_2CH_2$	H	CH_3	252–253	B	33	$C_{13}H_{16}ClNO_4$	54.65	5.65	4.90	54.79	5.54	5.09
18	4-$ClC_6H_4CH_2CH_2$	H	CH_3	240–241	B	78	$C_{13}H_{16}ClNO_4$	54.65	5.65	4.90	54.41	5.74	4.85
19	3,4-$(CH_3O)_2C_6H_3CH_2CH_2$	H	CH_3	229–230	A	31	$C_{15}H_{21}NO_6$	57.87	6.80	4.50	57.98	6.92	4.76
20	$C_6H_5CH_2CH(CH_3)$	H	CH_3	190–191	E	46	$C_{14}H_{19}NO_4$	63.38	7.22	5.28	63.42	7.15	5.62
21	$(C_6H_5)_2CHCH_2$	H	CH_3	145–147	E	67	$C_{19}H_{21}NO_4$	69.70	6.47	4.28	69.62	6.49	4.05
22	3-$C_5H_4NCH_2$[b]	H	CH_3	167–168	E	73	$C_{11}H_{14}N_2O_4$	55.45	5.92	11.76	55.33	5.73	11.90
23	4-C_5H_4N[b]	H	CH_3	217–218 dec	E	67	$C_{10}H_{12}N_2O_4$	53.57	5.39	12.50	53.64	5.37	12.60
24	$C_4H_8NCH_2CH_2$[d]	H	CH_3	167–169	D	25	$C_{11}H_{20}N_2O_4$	54.08	8.25	11.47	54.38	8.26	11.62
25	$C_8H_{11}N_2CH_2CH_2$[e]	H	CH_3	193–195	G	82	$C_{12}H_{23}N_3O_4$	52.73	8.48	15.37	52.83	8.51	15.00
26	$-(CH_2)_4-$		CH_3	152–153	H	69	$C_9H_{15}NO_4$	53.72	7.51	6.96	53.75	7.47	6.73
27	$-CH_2CH_2(CH_3)NCH_2CH_2-$		CH_3	183–184	E	75	$C_{10}H_{18}N_2O_4$	52.16	7.88	12.17	51.96	7.98	12.49

[a] A = water, B = dimethylformamide, C = ethanol + water, D = ethanol, E = acetone + water, F = Methyl Cellosolve, G = isopropyl alcohol + acetone, H = *n*-propyl alcohol + hexane. [b] C_5H_4N = pyridyl. [c] C_6H_{11} = cyclohexyl. [d] C_4H_8N = pyrrolidino. [e] $C_6H_{11}N_2$ = N-methylpiperazino.

N-Substituted DL-Aspartic Acids.—The specific procedure of Zilkha and Bachi[4] was employed and the resulting product was recrystallized from a suitable solvent. The new acids prepared are listed in Table I.

N-[(4-Tolylsulfonyl)carbamoyl]amino Acids

MARVIN J. KARTEN,[1] ALLAN SCHWINN, AND WILLIAM OROSHNIK

U. S. Vitamin and Pharmaceutical Corporation, Yonkers, New York 10701

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In our search for potential hypoglycemic agents, we were led to investigate the report by Bramanti and Di Paco[2] of possible hypoglycemic activity of N-[(4-tolylsulfonyl)carbamoyl]leucine. We wish to report the synthesis of some new N-[(4-tolylsulfonyl)-carbamoyl]amino acids. Prior synthesis[2] of such compounds involved the reaction of the ethyl esters of leucine and α-aminobutyric acid with 4-tolylsulfonyl isocyanate or 4-tolylsulfonylurethan, followed by alkaline hydrolysis. The reaction of 4-tolylsulfonylurea with amines in acetic acid and with glycine ethyl ester hydrochloride as a melt to give N-substituted N′-tolylsulfonylureas has also been reported.[3] We have found that heating 4-tolylsulfonylurea with amino acids in acetic acid affords the title compounds in fair yield. These results are summarized in Table I. The compounds showed no hypoglycemic activity.

Experimental Section[4]

4-Tolylsulfonylurea was prepared according to Kharag, Yavlinskii, and Savin.[5] Compounds **2–5** were prepared using the DL-amino acids. Compounds **6–8** were prepared using the L-amino acids. The optical purity of the compounds was not determined.

N-[(4-Tolylsulfonyl)carbamoyl]amino Acids.—The following example is typical of the method of preparation of compounds **1–5**, **7**, and **9** in Table I. A mixture of 0.04 mole of 4-tolylsulfonylurea, 0.08 mole of the amino acid, and 60 ml of glacial acetic acid was heated at 90–100° for 5.0 hr. After cooling, the reaction mixture was poured into 400 ml of water and refrigerated to complete precipitation of the product. The product was filtered, washed with water, dissolved in the minimum amount of 1 *N* Na_2CO_3, and filtered. Acidification of the filtrate with 3 *N* HCl gave a solid which was dried and recrystallized from a suitable solvent. With the exception of **6**, the compounds in Table I gave a negative ninhydrin[6a] test. Compound **7** also gave a positive Sakaguchi[6b] test.

N^6-Benzyloxycarbonyl-N^2-[(4-tolylsulfonyl)carbamoyl]lysine.—A mixture of N^6-benzyloxycarbonyl-L-lysine[7a] (0.04 mole, 11.2 g), 4-tolylsulfonylurea (0.04 mole, 8.56 g), and 100 ml of glacial acetic acid was heated at 90–100° for 6 hr, poured into 500 ml of water, and refrigerated. The gummy solid was filtered and dissolved in acetone, the solution was filtered, and the filtrate was evaporated *in vacuo* to give an oil. The oil was dissolved in a saturated $KHCO_3$ solution, the solution was filtered, and the filtrate was acidified with 3 *N* HCl to give a gum. The gum was washed with water, dried, dissolved in a minimum amount of ethyl acetate, and treated with petroleum ether (bp 30–60°) to give an amorphous solid which, after drying, weighed 9.3 g. An

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