REFERENCES

(1) Teorell, T., *Arch. Intern. Pharmacodyn.*, **57**, 205 (1937).
(2) Dominguez, R., "Kinetics of Elimination, Absorption and Volume of Distribution in the Organism," Medical Physics II, Yearbook Publishers, Inc., Chicago, Ill., 1950, pp. 476–489.
(3) Dost, F. H., "Der Blutspiegel," Thieme, Leipzig, 1953.
(4) Nelson, E., *J. Pharm. Sci.*, **50**, 181(1961).
(5) Wagner, J. G., *ibid.*, **50**, 359(1961).
(6) Krüger-Thiemer, E., *J. Theoret. Biol.*, **13**, 212(1966).
(7) Way, E. L., and Adler, T. K., *Bull. World Health Organ.*, **27**, 359(1962).
(8) Wagner, J. G., *J. Pharm. Sci.*, **52**, 1097(1963).
(9) Martin, B. K., *Brit. J. Pharmacol.*, **29**, 181(1967).
(10) Heimlich, K. R., MacDonnell, D. R., Flanagan, T. L., and O'Brien, P. D., *J. Pharm. Sci.*, **50**, 232(1961).
(11) Beckett, A. H., and Wilkinson, G. R., *J. Pharm. Pharmacol.*, **17**, 107S(1965).
(12) Wilkinson, G. R., and Beckett, A. H., *J. Pharmacol. Exptl. Therap.*, **162**, 139(1968).
(13) Jackson, A. S., "Analog Computation," McGraw-Hill, New York, N. Y., 1960.
(14) Wagner, J. G., and Nelson, E., *J. Pharm. Sci.*, **52**, 610(1963).
(15) Riegelman, S., Loo, J., and Rowland, M., *ibid.*, **57**, 117(1968).
(16) Wilkinson, G. R., and Beckett, A. H., in press.
(17) Beckett, A. H., and Tucker, G. T., *J. Pharm. Pharmacol.*, **18**, 72S(1966).

Keyphrases

Ephedrines—pharmacokinetics
Pharmacokinetics—ephedrines, absorption, metabolism, excretion
Models, single compartment—ephedrines, pharmacokinetics
Metabolites, ephedrines—formation, elimination

Stilbene Isothiocyanates as Potential Fluorescent Tagging Agents

By J. E. SINSHEIMER, J. T. STEWART*, and J. H. BURCKHALTER

Two groups of stilbene isothiocyanate derivatives, α-phenylcinnamic acids and α-phenylcinnamonitriles, have been synthesized. These compounds and their required intermediates were studied as to their structure-fluorescence relationships in an effort to develop a protein-tagging agent with blue fluorescence.

THE FLUORESCENT labeling of proteins as first developed by Coons *et al.* (1) and advanced by the introduction of fluorescent isothiocyanates by Burckhalter and co-workers (2, 3) has had extensive application to biological problems especially for the identification of pathogenic organisms. The agents and techniques employed together with their applications have been reviewed by Nairn (4). In varying degrees, all the presently available reagents can be improved upon in regard to purity, expense, fluorescent intensity, and stability to UV irradiation. It would also be highly desirable to have agents with contrasting fluorescent colors. For example, a blue fluorescent label would be valuable to supplement the green of fluorescein and orange to red fluorescence of rhodamine derivatives.

Limited studies with blue fluorescent tagging agents have employed stilbene optical brightening agents and a coumarin isocyanate (5) as well as β-anthryl isocyanate (6) with limited success.

Peck and Creech (7) synthesized 4′-isocyanato-4-dimethylaminostilbene for the purpose of combining it with proteins for UV analysis, but no mention was made of the possibility of utilizing the blue fluorescent properties of the stilbene and resulting protein conjugate. A blue fluorescent reagent, the disodium salt of 4′-acetamido-4-isothiocyanatostilbene-2,2′-disulfonic acid, has been described for the specific labeling of the outer components of the plasma membrane, but it has not been applied to antibody labeling (8).

Successful use of stilbene compounds as blue fluorescent optical brightening agents (9) of high stability encouraged the authors to seek improved fluorescent labeling agents among stilbene derivatives. The purpose of this investigation was to synthesize a stilbene isothiocyanate suitable for protein labeling as well as to study structure-fluorescence relationships in a series of model stilbene compounds. More specifically a stilbene isothiocyanate protein-tagging agent was postu-

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lated to meet the needs for an agent of good stability when exposed to UV radiation and for a blue fluorescent label as a compliment to the existing red and green agents. A contrasting color was desired for such applications as multiple antigen identification and as an additional method of overcoming background color interference.

DISCUSSION

Primarily two groups of stilbene isothiocyanate derivatives, α-phenylcinnamic acids and α-phenylcinnamonitriles, were synthesized and evaluated as labeling agents. The inclusion of the carboxylic group in the case of the former series of compounds was to promote water solubility of these compounds as their sodium salts for ultimate use as protein labels in aqueous media. Even more extensive studies were conducted with the α-phenylcinnamonitriles. The literature indicated (10, 11), including personal experiences (12, 13), that these compounds possessed good fluorescent intensity in solution by visual detection. An added reason for choosing the nitriles is that the synthetic route to these compounds produces primarily *trans* stilbenes; it is noteworthy that *cis* stilbene possesses only 1% of the fluorescence of its *trans* isomer (14).

The fluorescent-enhancing effect of various constituents was of interest. Such groups, however, could not be reactive with isothiocyanates and, therefore, studies were restricted to the effects of methoxy, dimethylamino, and quaternary nitrogen substituents. The isothiocyanates were also reacted with benzylamine to form thioureas corresponding to the thiourea linkage of a conjugated protein and to observe the effect of this structure on fluorescence.

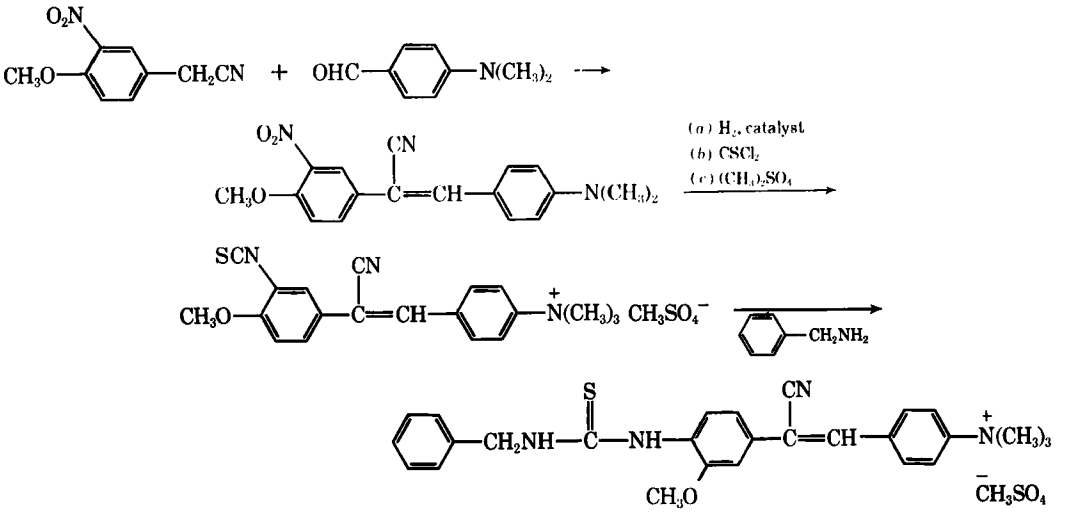
A typical synthetic route to a quaternary salt of an α-phenylcinnamonitrile and conversion to the thiourea is shown in Scheme I.

The α-phenylcinnamic acids were synthesized *via* base-catalyzed condensations of substituted phenylacetic acids and benzaldehydes (15, 16). Starting materials required for the synthesis were either available commercially or could be readily obtained in a few synthetic steps from commercial materials.

The isothiocyanates, thioureas, and new intermediates which were synthesized are shown together with their physical constants and fluorescence characteristics in Table I. This table also includes three stilbenes synthesized for purpose of comparison by methods analogous to known base condensation procedures (17–19).

Initially, measurement of the fluorescence of compounds with emphasis on isothiocyanate and thiourea compounds was by comparison to quinine sulfate by quinine reference unit (QRU) (20) with use of a filter fluorometer. Fluorescent values of aqueous solutions (ethanolic if required) were obtained when possible at a concentration where fluorescence was shown to be proportional to concentration. Compounds for which a linear concentration fluorescence relationship could not be obtained are indicated in Table I. For all compounds, fluorescence measurements were made as suggested by Hercules (21) at concentrations whose absorbance was less than 0.05 at the excitation wavelength. The fluorometer filters were selected for activation at 365 mμ (Corning 7-37 filter) and for detection of emitted light at wavelengths greater than 400 mμ (Kodak 2-A filter). These filters are similar to those commercially available in fluorescent microscopy and are suitable for the observation of blue fluorescence.

Structure-fluorescence relationships were obtained from the QRU data and comparisons of the data in Table I can be made to show the effect of different groups on the fluorescence of a molecule. In a limited study, α-phenylcinnamic acids were found to be less fluorescent than α-phenylcinnamonitriles, which possess a weaker fluorescence than stilbenes containing identical substitution. Methoxy and isothiocyanato substituents in α-phenylcinnamonitriles result in increased fluorescent intensity. The increase depended on the fluorescent species involved. Thiourea derivatives formed from isothiocyanates of the α-phenylcinnamonitrile series possess a weaker fluorescent intensity than corresponding isothiocyanates. This is in contrast



Scheme I

Table I—Stilbene Derivatives

Compd.	R_1	R_2	M.p., °C.	Method of Synthesis[a]	Yield, %	Anal. Calcd.	Anal. Found	UV $\lambda_{max.}$ mμ	IR $\nu_{max.}$ cm.$^{-1}$	Fluorescence Excit. $\lambda_{max.}$	Fluorescence Emiss. $\lambda_{max.}$	Relative Fluorescence QRU[r]
				A. α-Phenylcinnamic Acids								
1	*cis*-4-Nitrophenyl	4-Dimethylaminophenyl	268–269		83	C, 65.38 H, 5.16 N, 8.97	65.39 5.30 8.94	320 (ε 31,200)	1,660 (C=O) 1,600 (phenyl) 1,320 (NO_2)	—	—	—
2	*cis*-4-Aminophenyl	4-Dimethylaminophenyl	228–230	A	81	C, 72.32 H, 6.43	72.34 6.49	360 (ε 6,690) 280 (ε 9,080)	3,300 (NH) 1,650 (C=O) 1,600 (phenyl)	—	—	—
3	*cis*-4-Isothiocyanatophenyl	4-Dimethylaminophenyl	225–227	B	80	C, 66.64 H, 4.94 N, 8.64	66.73 5.05 8.56	360 (ε 33,000)	2,100 (NCS) 1,640 (C=O) 1,575 (phenyl)	—	—	0.001
4	*trans*-4-Aminophenyl	4-Dimethylaminophenyl	225–226	A[b]	85	C, 72.32 H, 6.43 N, 9.92	71.96 6.35 9.80	360 (ε 25,900) 240 (ε 10,600)	3,300 (NH) 1,670 (C=O) 1,600 (phenyl)	—	—	—
5	*trans*-4-Isothiocyanatophenyl	4-Dimethylaminophenyl	221–222	B	94	C, 66.64 H, 4.94 N, 8.64	66.73 4.90 8.58	353 (ε 24,100)	2,100 (NCS) 1,650 (C=O) 1,575 (phenyl)	—	—	0.000[s]
6	*cis*-4-Methoxyphenyl	4-Aminophenyl	223–226	A[c]	17	—	—[q]	—	1,625 (C=O) 1,550 (phenyl)	—	—	—
7	*cis*-4-Methoxyphenyl	4-Isothiocyanatophenyl	144–146	B	50	C, 65.58 H, 4.21 N, 4.50	65.30 4.44 4.46	333 (ε 17,000)	2,020 (NCS) 1,700 (C=O) 1,500 (phenyl) 1,240 (arom. ether)	—	—	0.000[s]
8	*trans*-4-Methoxyphenyl	4-Aminophenyl	205–208	A[c]	60	C, 71.36 H, 5.61 N, 5.20	71.21 5.61 5.30	330 (ε 10,500)	1,650 (C=O) 1,575 (phenyl) 1,250 (arom. ether)	—	—	—
9	*trans*-4-Methoxyphenyl	4-Isothiocyanatophenyl	156–158	B	80	C, 65.58 H, 4.21 N, 4.50	65.77 4.29 4.69	328 (ε 19,100)	2,100 (NCS) 1,675 (C=O) 1,600 (phenyl) 1,275 (arom. ether)	—	—	0.000
10	*cis*-4-Methoxyphenyl	3-Nitrophenyl	199–202	[c]	8.4	C, 64.21 H, 4.38 N, 4.68	64.28 4.48 4.71	305 (ε 25,500)	1,700 (C=O) 1,340; 1,520 (NO_2) 1,600 (phenyl)	—	—	—
11	*cis*-4-Methoxyphenyl	3-Aminophenyl	58–60(dec.)	A	51	—	—[q]	—	—	—	—	—
12	*cis*-4-Methoxyphenyl	3-Isothiocyanatophenyl	135–138	B	96	C, 65.58 H, 4.21 N, 4.50	65.52 4.33 4.50	218 (ε 33,200)	2,100 (NCS) 1,700 (C=O) 1,600; 1,520 (phenyl) 1,250 (arom. ether)	—	—	0.0004[t]
13	*trans*-4-Methoxyphenyl	3-Nitrophenyl	174–176	[c]	55	C, 64.21 H, 4.38 N, 4.68	64.31 4.42 4.70	225 (ε 21,000) 260 (ε 19,700)	1,650 (C=O) 1,600 (phenyl) 1,520; 1,340 (NO_2)	—	—	—
14	*trans*-4-Methoxyphenyl	3-Aminophenyl	222–224	A	70	C, 71.36 H, 5.61 N, 5.20	70.66 5.70 5.16	225 (ε 21,800) 280 (ε 13,100)	1,660 (C=O) 1,560 (phenyl) 1,225 (arom. ether)	—	—	—
15	*trans*-4-Methoxyphenyl	3-Isothiocyanatophenyl	148–151	B	90	C, 65.58 H, 4.21 N, 4.50	65.60 4.34 4.63	221 (ε 22,300)	2,100 (NCS) 1,660 (C=O) 1,560; 1,500 (phenyl) 1,250 (arom. ether)	—	—	0.0003[t]

B. α-Phenylcinnamonitriles $R_1-C(CN)=CH-R_2$

16	4-Methoxyphenyl	3-Isothiocyanatophenyl	109–110	B[d]	89	C, 69.84 H, 4.14 N, 9.58	69.86 4.01 9.55	222 (ε 21,800)	2,200 (CN) 2,500 (NCS)	350	425	0.070
17	4-Methoxyphenyl	[3-(3-Benzylthioureido)-phenyl]	185–187	D	81	C, 72.15 H, 5.30 N, 10.52	71.89 5.38 10.52	240 (ε 30,000)	2,250 (CN) 1,550; 1,520 (phenyl)	365	490	0.020
18	4-Methoxyphenyl	2-Methoxy-5-nitro-phenyl	150–152	e,f	93	C, 65.80 H, 4.55 N, 9.03	65.87 4.64 9.07	232 (ε 16,500) 305 (ε 22,000) 335 (ε 21,400)	2,200 (CN) 1,575; 1,560 (phenyl) 1,500; 1,325 (NO_2) 1,250 (arom. ether)	—	—	—
19	4-Methoxyphenyl	2-Methoxy-5-amino-phenyl	146–149	A	62	C, 72.84 H, 5.75 N, 9.99	72.82 5.73 10.00	325 (ε 17,000)	2,200 (CN) 1,590 (phenyl) 1,225 (arom. ether)	400	570	0.007
20	4-Methoxyphenyl	2-Methoxy-5-trimethyl-ammoniumphenyl iodide	183–185	C	71	C, 53.34 H, 5.15 N, 6.22	53.30 5.22 6.26	340 (ε 16,000)	2,275 (CN) 1,540; 1,520 (phenyl)	355	440	0.040
21	4-Methoxyphenyl	2-Methoxy-5-isothio-cyanatophenyl	168–172	B	60	C, 67.06 H, 4.38 N, 8.69	67.16 4.43 8.67	225 (ε 29,300) 285 (ε 30,900)	2,200 (CN) 2,100 (NCS) 1,600; 1,500 (phenyl) 1,240 (arom. ether)	400	475	0.016
22	4-Methoxyphenyl	[2-Methoxy-5-(3-benzylthioureido)-phenyl]	157–158	D	70	C, 69.91 H, 5.40 N, 9.78	70.00 5.53 9.58	240 (ε 29,000) 320 (ε 11,100)	2,250 (CN) 1,625; 1,550 (phenyl)	370	500	0.013
23	4-Isothiocyanatophenyl	Styryl	128–130	B[g]	86	C, 74.97 H, 4.20 N, 9.72	74.84 4.20 9.56	285 (ε 21,600) 350 (ε 31,400)	2,200 (CN) 2,060 (NCS) 1,600 (phenyl)	400	465	0.010
24	[4-(3-Benzylthioureido)-phenyl]	Styryl	225–227	D	75	C, 75.92 H, 5.35 N, 10.62	75.66 5.41 10.75	245 (ε 24,500) 355 (ε 34,100)	2,250 (CN) 1,570 (phenyl)	400	465	0.050
25	4-Isothiocyanatophenyl	Phenyl	111–113	B[g]	90	C, 73.26 H, 3.84 N, 10.68	73.17 3.74 10.57	330 (ε 44,200)	2,275 (CN) 2,150 (NCS) 1,540 (phenyl)	380	570	0.003
26	[4-(3-Benzylthioureido)-phenyl]	Phenyl	202–204	D	83	C, 74.77 H, 5.18 N, 11.37	74.62 5.02 11.50	240 (ε 21,000) 340 (ε 24,900)	2,275 (CN) 1,610; 1,575 (phenyl)	360	510	0.0045[t]
27	Phenyl	3,4-Dimethoxyphenyl	87–88	f	80	C, 76.96 H, 5.70 N, 5.28	77.00 5.63 5.26	348 (ε 20,000)	2,200 (CN) 1,575; 1,500 (phenyl) 1,260 (arom. ether)	—	—	0.003[t]
28	4-Methoxyphenyl	2,3-Dimethoxyphenyl	90–93		90	C, 73.20 H, 5.80 N, 4.74	72.96 5.82 4.80	330 (ε 20,800) 225 (ε 17,800)	2,250 (CN) 1,615; 1,600 (phenyl) 1,275 (arom. ether)	—	—	0.003[t]
29	4-Hydroxyphenyl	4-Dimethylaminophenyl	228–232		50	C, 77.25 H, 6.10 N, 10.60	77.07 6.12 10.54	250 (ε 11,400) 375 (ε 22,400)	2,200 (CN) 1,590; 1,550 (phenyl)	365	450	0.005
30	4-Benzyloxyphenyl	4-Dimethylaminophenyl	134–136		80	C, 81.33 H, 6.26 N, 7.90	81.12 6.39 7.88	250 (ε 11,400) 380 (ε 21,000)	2,280 (CN) 1,640; 1,600 (phenyl)	365	440	0.009[t]
31	4-Benzyloxyphenyl	4-Trimethylammonium-phenyl iodide	166–168	C	90	C, 60.49 H, 5.08 N, 5.64	60.89 5.16 5.80	335 (ε 13,100)	2,250 (CN) 1,625; 1,525 (phenyl)	350	440	0.060
32	4-Butoxyphenyl	4-Dimethylaminophenyl	114–116		83	C, 78.71 H, 7.55 N, 8.74	78.59 7.45 8.78	375 (ε 36,400) 283 (ε 17,600) 252 (ε 24,400) 228 (ε 26,100)	2,175 (CN) 1,600 (phenyl)	270	450	0.002[t]

(Continued on next page.)

TABLE I (*Continued.*)

Compd.	R_1	R_2	M.p., °C.	Method of Synthesis[a]	Yield, %	Anal. Calcd.	Anal. Found	UV $\lambda_{max.}$ mμ	IR $\nu_{max.}$ cm.$^{-1}$	Fluorescence Excit. λ_{max}	Fluorescence Emiss. $\lambda_{max.}$	Relative Fluorescence QRU[r]
33	4-Butoxyphenyl	4-Trimethylammonium-phenyl iodide	155–157	C	80	C, 57.15 H, 5.89 N, 6.06	57.26 5.82 6.10	330 (ε 26,500)	2,275 (CN) 1,640; 1,600 (phenyl)	350	440	0.070
34	3,4-Dimethoxyphenyl	4-Dimethylaminophenyl	107–109	[f]	90	C, 74.00 H, 6.54 N, 9.08	73.94 6.53 9.17	380 (ε 30,000) 250 (ε 17,100)	2,200 (CN) 1,600 (phenyl)	280	450	0.011
35	3,4-Dimethoxyphenyl	4-Trimethylammonium-phenyl iodide	173–175	C	71	C, 53.34 H, 5.15 N, 6.22	53.30 5.23 6.21	350 (ε 11,700) 265 (ε 7,980) 255 (ε 7,670)	2,250 (CN) 1,660; 1,620 (phenyl)	365	490	1.750
36	3,4,5-Trimethoxyphenyl	4-Dimethylaminophenyl	169–170		88	C, 70.99 H, 6.55 N, 8.28	70.89 6.48 8.34	380 (ε 31,200) 255 (ε 15,600)	2,200 (CN) 1,600 (phenyl) 1,250 (arom. ether)	365	470	0.006[t]
37	3,4,5-Trimethoxyphenyl	4-Trimethylammonium-phenyl iodide	167–169	C	71	C, 52.51 H, 5.25 N, 5.83	52.29 5.36 5.84	340 (ε 18,000) 260 (ε 13,000)	2,275 (CN) 1,650; 1,610 (phenyl); 1,275 (arom. ether)	350	500	0.840
38	4-Methoxyphenyl	3-Trimethylammonium-phenyl iodide	164–168	C[d]	48	C, 54.30 H, 5.04 N, 6.67	54.36 5.11 6.62	330 (ε 15,200)	2,200 (CN) 1,590; 1,500 (phenyl) 1,235 (arom. ether)	350	440	0.200
39	4-Methoxyphenyl	4-Trimethylammonium-phenyl iodide	173–175	C[i]	60	C, 54.30 H, 5.04 N, 6.67	54.46 4.99 6.71	320 (ε 14,400) 230 (ε 27,600)	2,200 (CN) 1,590; 1,500 (phenyl) 1,235 (arom. ether)	350	425	0.150
40	3-Nitro-4-methoxy-phenyl	4-Dimethylaminophenyl	211–212	[k,l]	92	C, 66.86 H, 5.30 N, 13.00	67.10 5.41 12.90	395 (ε 26,100) 255 (ε 15,100)	2,200 (CN) 1,600; 1,560 (phenyl) 1,520; 1,340 (NO_2)	—	—	—
41	3-Amino-4-methoxy-phenyl	4-Dimethylaminophenyl	177–179	A	88	C, 73.69 H, 6.53 N, 14.32	73.63 6.48 14.40	375 (ε 28,000) 250 (ε 22,300)	3,500 (NH) 2,200 (CN) 1,590; 1,575 (phenyl)	365	440	0.007[t]
42	3-Isothiocyanato-4-methoxyphenyl	4-Dimethylaminophenyl	139–141	B	88	C, 68.03 H, 5.11 N, 12.53	68.00 5.28 12.41	385 (ε 35,600) 255 (ε 27,300)	2,200 (CN) 2,050 (NCS) 1,600; 1,575 (phenyl)	420	475	0.013[t]
43	3-Isothiocyanato-4-methoxyphenyl	4-Trimethylammonium-phenyl methosulfate	192–196	C	90	—	—[e]	275 (ε 19,100) 215 (ε 39,800)	2,200 (CN) 2,050 (NCS) 1,600 (phenyl) 1,250 (arom. ether)	350	450	0.300
44	3-(3-Benzylthioureido)-4-methoxyphenyl	4-Trimethylammonium-phenyl methosulfate	168–170	D	80	C, 59.13 H, 5.67 N, 9.85	59.03 5.61 9.75	250 (ε 25,100)	2,200 (CN) 1,600 (phenyl) 1,240 (arom ether)	365	460	0.020
45	4-Methoxyphenyl	2-Dimethylamino-5-nitrophenyl	174–175	[m,n]	94	C, 66.86 H, 5.30 N, 13.00	66.92 5.31 12.85	370 (ε 15,200) 325 (ε 16,900)	2,300 (CN) 1,650 (phenyl) 1,520; 1,350 (NO_2)	—	—	—
46	4-Methoxyphenyl	2-Dimethylamino-5-aminophenyl	109–111	A	83	C, 73.69 H, 6.53 N, 14.32	73.73 6.52 14.30	325 (ε 14,200) 240 (ε 13,600)	2,250 (CN) 1,515 (phenyl) 1,260 (arom. ether)	—	—	—
47	4-Methoxyphenyl	2-Dimethylamino-5-isothiocyanatophenyl	95–97	B	87	C, 68.03 H, 5.11 N, 12.53	68.17 4.96 12.49	305 (ε 27,400) 235 (ε 20,800)	2,250 (CN) 2,150 (NCS) 1,650 (phenyl)	335	550	0.013

48	4-Methoxyphenyl	[2-Dimethylamino-3-(3-benzylthioureido)-phenyl]	178–181	D	80	C, 70.56 H, 5.92 N, 12.66	70.36 5.79 12.56	285 (ε 21,800) 240 (ε 25,400)	2,250 (CN) 1,575; 1,515 (phenyl) 1,260 (arom. ether)	335	540	0.017
49	4-Aminophenyl	4-Dimethylaminophenyl	194–196	A[b]	56	C, 77.54 H, 6.51 N, 15.96	77.55 6.48 16.01	380 (ε 25,200) 250 (ε 17,500)	3,300 (NH) 2,200 (CN) 1,550 (phenyl)	420	480	—
50	4-Isothiocyanatophenyl	4-Dimethylaminophenyl	129–131	B	80	C, 70.78 H, 4.95 N, 13.75	70.83 5.03 13.72	292 (ε 17,900)	2,250 (CN) 2,150 (NCS) 1,640; 1,600 (phenyl)	365	460	0.008
51	[4-(3-Benzylthioureido)-phenyl]	4-Dimethylaminophenyl	190–192	D	60	C, 72.78 H, 5.86 N, 13.58	72.71 5.91 13.60	385 (ε 27,500) 250 (ε 24,000)	2,250 (CN) 1,640; 1,600 (phenyl)	390	460	0.005
				C. Stilbenes R_1—CH=CH—R_2								
52	4-Dimethylaminophenyl	4-Isothiocyanatophenyl	214–216	B[c,p]	90	C, 72.82 H, 5.75 N, 9.99	72.59 5.70 10.09	375 (ε 40,600) 285 (ε 14,500)	2,150 (NCS) 1,640; 1,550 (phenyl)	410	470	0.040
53	4-Dimethylaminophenyl	4-(3-Benzylthioureido)-phenyl	193–194	D	74	C, 74.38 H, 6.50 N, 10.84	74.51 6.50 10.78	355 (ε 30,900)	1,640; 1,550 (phenyl)	390	450	0.100

[a] Subscripts are for general literature procedures and key intermediates. Letters refer to the authors' experimental procedures. [b] The *cis* acid was prepared from the *cis* ethyl ester reported by Pfeiffer (23) with the same general procedure used by Pfeiffer for his preparation of the *trans* acid. [c] See Ref. 15 for general synthetic procedure. [d] See Ref. 13. [e] See Ref. 24. [f] Synthesized according to general procedure described in Ref. 25. [g] See Ref. 11. [h] Synthesized from Compd. 28 by demethylation with hydrobromic-acetic acids. [i] Synthesized from Compd. 29 by general procedure described in Ref. 26. [j] See Ref. 27. [k] See Ref. 28. [l] See Ref. 29. [m] See Ref. 30. [n] See Ref. 31. [o] See Ref. 7. [p] See Ref. 32. [q] This compd. was employed as an intermediate to yield a derivative whose structure was supported by analytical data. [r] Quinine reference unit, see Ref. 20. [s] No fluorescence even at a concentration with an absorbance of 0.05 at 365 mμ. [t] Compound for which a linear concentration-fluorescence relationship could not be obtained.

TABLE II—FLUORESCENCE OF SELECTED COMPOUNDS RELATIVE TO FLUORESCEIN ISOTHIOCYANATE

Compd.	Relative QRU[a]	Fluorescein Isothiocyanate Excitation at 365 mμ (with Visual Detection Filter)	490 mμ Visual Detection Filter Absent	490 mμ Visual Detection Filter Present
Fluorescein isothiocyanate	1.000	1.000	1.000	1.000
Quinine sulfate	2.100	0.240	0.490	0.0150
43	0.640	0.060	0.067	0.0037
44	0.040	0.003	0.002	0.0003
16	0.150	0.006	0.029	0.0004
17	0.040	0.005	0.017	0.0003
52	0.090	0.006	0.0047	0.0004
53	0.220	0.011	0.022	0.0007
39	0.320	0.014	0.049	0.0009
35	3.720	0.540	0.354	0.0350
37	1.800	0.600	0.417	0.0391
33	0.150	0.012	0.0306	0.0008
31	0.130	0.013	0.023	0.0009
20	0.090	0.004	0.063	0.0003

[a] Quinine reference unit, see Ref. 20.

to an example studied in the stilbene series in which the thiourea is more fluorescent than the isothiocyanate.

The intense fluorescence of the quaternary salts of the α-phenylcinnamonitriles is of special interest. It was shown that they were more fluorescent in absolute ethanol than water.

The three isothiocyanates, Compounds 16, 43, and 52, that possessed QRU greater than 0.04 were tested in the fluorescent antibody procedure.[1] A QRU of 0.04 represents an intensity of a tenth that of fluorescein isothiocyanate when it is excited at 365 mμ. Conjugation of isothiocyanate 43 to rabbit antiserum proceeded as established for the commercial isothiocyanates (4). The water-insoluble Compounds 16 and 52 were dissolved in a small amount of acetone and then added to the serum. Although different excitation and barrier filter combinations available in the microscope were employed, no usable fluorescence was observed.[1]

An important factor in reference to these results is that the antibody-labeling technique involves visual detection of emitted fluorescence and that the compounds under study fluoresce in the blue region of the spectrum where the sensitivity of the human eye is relatively poor. As previously described (22), a fluorometer employing a Kodak wratten filter No. 106 with a 1P28 phototube will approach the spectral response of the eye. Emission spectra of the isothiocyanates that had been tested in the fluorescent antibody procedure, along with their thiourea derivatives and other compounds of interest from the synthetic studies, were determined with an Aminco-Bowman spectrophotofluorometer with the 106 filter, 1P28 phototube system and are listed in Table II. Solvents, pH, and activation wavelength were selected to yield the maximum fluorescence for each compound studied.

The relative fluorescence data in Table II indicate why Compounds 16, 43, and 52, that appeared promising in the QRU procedure, did not give

[1] We are indebted to Dr. W. C. Eveland and his staff in the University of Michigan School of Public Health for evaluating these compounds.

satisfactory results in the fluorescent antibody technique. The relative fluorescence in reference to fluorescein isothiocyanate activated at 365 mμ and detected by a system comparable to the spectral response of the eye was only one-tenth of corresponding relative values obtained by the QRU procedure. Each of these blue fluorescent compounds was reduced even further to a small fraction of the fluorescence of fluorescein isothiocyanate activated at its maximum of 490 mμ. The comparison on this latter basis is also listed in Table II.

Thus, while the objective of obtaining a contrasting blue fluorescence was met, the intensity of the compounds was not great enough to overcome the disadvantage of emission in a region where the sensitivity of the eye is limited. This is particularly unfortunate because these compounds are excited by the intense mercury lines near 365 mμ and are reasonably stable to ultraviolet irradiation, advantages not found in the tagging agents presently employed.

EXPERIMENTAL[2]

Reduction of Nitro to Amino Compounds (Procedure A)—A suspension or solution of a nitro compound and 100 mg. of Adams' catalyst/10 g. of compound in absolute ethanol was allowed to consume a calculated amount of hydrogen in a Parr hydrogenator. The catalyst was removed by filtration through diatomaceous earth[3] and the ethanol was evaporated. The residue can be recrystallized from aqueous ethanol or another appropriate solvent.

Formation of an Isothiocyanate from an Amino Compound (Procedure B)—An acetone solution of the amino compound and an excess of thiophosgene (hood) was refluxed on a steam bath for 2 hr. Removal under reduced pressure at room temperature of the unreacted thiophosgene (hood) and acetone leaves a material, which can be recrystallized from glacial or aqueous acetic acid.

Quaternary Salt Formation (Procedure C)—A suspension of 0.01 mole of amino compound, 0.03 mole of dimethyl sulfate, 5 ml. of water, and 5 g. of potassium carbonate in 60 ml. of acetone was heated on a steam bath for 2 hr. Filtration of the hot suspension followed by air evaporation of the filtrate (hood) gave the crude methosulfate salt. Addition of potassium iodide to an aqueous solution of the methosulfate formed the iodide which was collected by filtration and was dried. The methosulfate salts were recrystallized from absolute ethanol and the iodide salts were recrystallized from absolute ethanol and/or water.

Thiourea Formation (Procedure D)—An equimolar solution of an isothiocyanate compound and amine in absolute ethanol was heated in a water bath for 15–30 min. When the reaction mixture cooled to room temperature, the precipitate was collected and dried. Recrystallizations from absolute ethanol gave a pure thiourea.

Quinine Reference Unit Procedure—Quinine reference units (QRU) (20) were obtained on an Aminco fluoro-microphotometer. The samples were excited by means of a G.E. No. F4T4/B1 4 w. UV lamp and a Corning 7-37 filter. The detection system consisted of a Kodak wratten 2-A filter and a RCA 931-A phototube.

One milliliter of stock solution of quinine sulfate (99.3×10^{-6} g./ml. 0.1 N H_2SO_4) was diluted to 100 ml. with 0.1 N sulfuric acid, and a reading was obtained on the fluorometer.

Solutions of the fluorescent compounds (50 to 100×10^{-6} g./ml. in their appropriate solvents) were diluted and readings of the dilutions were taken until a linear relationship was observed between fluorescence and concentration.

Values of dilutions equivalent to absorbance units of no greater than 0.05 at the exciting wavelength of 365 mμ were employed to calculate the QRU when a linear relationship of fluorescence to concentration was not observed.

[2] Catalytic hydrogenation reactions were carried out at room temperature and 60 p.s.i. by means of a Parr hydrogenator. Melting points were taken in open capillary tubes with a Mel-Temp electric block; they are corrected. UV spectra were determined in ethanol solution by means of a Beckman model DB spectrophotometer. IR spectra were obtained with either a Perkin-Elmer Infracord or model 337 grating spectrophotometer. Fluorescence spectra were determined with an Aminco-Bowman spectrophotofluorometer model No. 4-8106 equipped with a X-Y recorder; spectra were uncorrected. Microanalyses were performed by Spang Microanalytical Laboratory, Ann Arbor, Mich.

[3] Celite 545, Johns-Manville Co., New York, N. Y.

REFERENCES

(1) Coons, A. H., Creech, H. J., Jones, R. N., and Berliner, E., *J. Immunol.*, **45**, 159(1942).

(2) Burckhalter, J. H., and Seiwald, R. J., U. S. pat. 2,937,186 (1960); through *Chem. Abstr.*, **54**, 19718(1960).

(3) Riggs, J. L., Seiwald, R. J., Burckhalter, J. H., Downs, C. M., and Metcalf, T. G., *Am. J. Pathol.*, **34**, 1081 (1958).

(4) Nairn, R. C., "Fluorescent Protein Tracing," 2nd ed., Williams and Wilkins, Baltimore, Md., 1964.

(5) Chadwick, C. S., McEntegart, M. G., and Nairn, R. C., *Immunology*, **1**, 317(1958).

(6) Creech, H. J., and Jones, R. N., *J. Am. Chem. Soc.*, **63**, 1661(1941).

(7) Peck, R. M., and Creech, H. J., *ibid.*, **74**, 468(1952).

(8) Maddy, A. H., *Biochim. Biophys. Acta*, **88**, 390 (1964).

(9) Adams, D. A. W. in "Recent Progress in Chemistry and Synthetic Colouring Matters and Related Fields," Gore, T. S., Joshi, B. S., Sunthankar, S. V., and Tilak, B. D., Eds., Academic Press, New York, N. Y., 1962, p. 615.

(10) Kauffmann, H., and Burr, K., *Ber.*, **40**, 2352(1907).

(11) Kauffmann, H., *ibid.*, **50**, 515, 1614(1917).

(12) Nerurkar, J. J., Ph.D. thesis, University of Kansas, Lawrence, Kan., 1959.

(13) Burckhalter, J. H., Sam, J., and Hall, L. A. R., *J. Am. Chem. Soc.*, **81**, 394(1959).

(14) Lewis, G. N., Magel, T. T., and Lipkin, D., *ibid.*, **62**, 2973(1940).

(15) Ketcham, R., and Jambotkar, D., *J. Org. Chem.*, **28**, 1034(1963).

(16) Johnson, J. R. "Organic Reactions," Vol. I, Wiley, New York, N. Y., 1942, p. 210.

(17) Pfeiffer, P., and Sergiewskaja, S., *Ber.*, **44**, 1107 (1911).

(18) Singh, R. N., Chand, V., and Choger, T. N., *Agra. Univ. J. Res. Science*, **1**, 153(1952); through *Chem. Abstr.*, **48**, 2654(1954).

(19) "Chemistry of Carbon Compounds," Vol. III, pt. B, Rodd, E. H., Ed., Elsevier, Amsterdam, 1956, p. 1138.

(20) Woods, L. L., and Sapp, J., *J. Chem. Eng. Data*, **8**, 235(1963).

(21) Hercules, D. L., *Anal. Chem.*, **38**, 29A(1966).

(22) Sinsheimer, J. E., Stewart, J. T., and Burckhalter, J. H., *J. Pharm. Sci.*, **57**, 333(1968).

(23) Pfeiffer, P., *Ann. Chem.*, **465**, 38(1926).

(24) Schnell, A., *Ber.*, **16**, 1381(1884).

(25) "Organic Syntheses," Coll. Vol. III, Wiley, New York, N. Y., 1955, p. 715.

(26) Miyano, M., Muraki, S. Kusunoki, T., Morita, T., and Matsui, M., *Nippon Nogeikagaku Kaishi*, **34** (8), 683 (1960); through *Chem. Abstr.*, **59**, 13929*c*(1963).

(27) Niederl, J. B., and Ziering, A., *J. Am. Chem. Soc.*, **64**, 885(1942).

(28) Quelet, R., and Germain, Y., *Compt. Rend.*, **202** 1442(1936).

(29) Bersch, H. W., *Arch. Pharm.*, **277**, 271(1939).

(30) Erdmann, H., *Ann. Chem.*, **272**, 153(1893).

(31) Cohn, P., and Blau, A., *Monatsch. Chem.*, **25**, 371 (1904).

(32) Pfeiffer, P., *Ber.*, **48**, 1796(1915).

Keyphrases

Stilbene isothiocyanates—synthesis
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Fluorometry—analysis

Dissolution Behavior and Solubility of Anhydrous and Trihydrate Forms of Ampicillin

By JOHN W. POOLE and CHANDER KANTA BAHAL

Anhydrous ampicillin and ampicillin trihydrate were compared for solubility and relative rates of dissolution in distilled water at temperatures ranging from 7.5 to 50°. Differences were noted in the physical-chemical properties of these two forms of ampicillin. The thermodynamic properties of these compounds have been experimentally evaluated. The properties noted for the two forms of the antibiotic are consistent with the observed differences in the biological utilization of the two forms after oral administration to laboratory animals and human subjects.

MANY ORGANIC medicinal compounds are capable of existing in more than one crystalline form having different physical-chemical properties. The resulting variation in the thermodynamic properties associated with differences in crystal form may be of considerable pharmaceutical importance as pointed out previously by Higuchi (1). The present report is concerned with studies conducted to determine the differences in some of the physical-chemical properties of two forms of ampicillin, a semisynthetic penicillin. Specifically, the solubilities and relative rates of dissolution in distilled water of anhydrous ampicillin and ampicillin trihydrate were determined and the thermodynamic properties of these crystal forms were experimentally evaluated.

Most of the past work reported on the physical-chemical properties of crystalline hydrates has been concerned with inorganic compounds. The studies of Taylor and Henderson (2) on the various hydrates of calcium nitrate and of Hill (3) on calcium sulfate are examples of such studies. More recently several investigations concerned with studies of organic molecules in the anhydrous and hydrated forms have been reported. An anhydrous form of phenobarbital and two of its hydrates were examined by Eriksson (4) for apparent solubility in water as a function of time. The relative dissolution rates of solvated and nonsolvated crystal forms of several types of compounds of pharmaceutical interest, including steroids and xanthines were reported by Shefter and Higuchi (5). These workers also determined the thermodynamic properties of several of these crystal systems.

EXPERIMENTAL

Apparatus—A constant-temperature water bath equipped with Unitherm Haake constant-temperature circulator[1] and a rotating-bottle apparatus,[2] Swinney hypodermic adaptor,[3] Millipore filters[3] (pore size 0.45 μ), amber bottles, 120 ml. with polyseal caps.[4]

Compounds—In all the experiments anhydrous ampicillin, (Wyeth Laboratories batch C-10575, m.p. 203–204°) was used. The trihydrate form of ampicillin was prepared from the anhydrous form by the method of Austin *et al.* (6). IR spectra and differential thermal analysis curves were obtained for this material.

Procedure—An excess of drug, 2 g., in the appropriate form was added to 100 ml. of distilled water previously equilibrated to the desired temperature.

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[1] Brinkmann Instruments, Westbury, N. Y.
[2] E. D. Menold Sheet Co., Lester, Pa.
[3] Millipore Corp., Bedford, Mass.
[4] Erno Products, Philadelphia, Pa.