Irreversible Enzyme Inhibitors. CXII.[1,2] Active-Site-Directed Irreversible Inhibitors of Dihydrofolic Reductase Derived from 4,6-Diamino-1,2-dihydro-2,2-dimethyl-1-phenyl-*s*-triazine Substituted with a Terminal Sulfonyl Fluoride. II[3]

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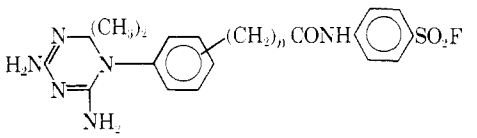
N-[*p*-(4,6-Diamino-1,2-dihydro-2,2-dimethyl-*s*-triazine-1-yl)-β-phenylpropionyl]sulfanilyl fluoride (**3**) and N-[*p*-(4,6-diamino-1,2-dihydro-2,2-dimethyl-*s*-triazine-1-yl)benzoyl]sulfanilyl fluoride (**4**) have been previously reported[3] to be active-site-directed irreversible inhibitors of the dihydrofolic reductases from pigeon liver, Walker 256 rat tumor, and L1210/FR8 mouse leukemia. Nine analogs have now been synthesized where the position of the sulfonyl fluoride group with respect to the 1-phenyl-*s*-triazine moiety has been varied; these have been evaluated on the two tumor enzymes. The *meta* isomer **5** of **3** was still an effective irreversible inhibitor of the Walker 256 enzyme but was no longer an irreversible inhibitor of the enzyme from L1210/FR8. In contrast, the *meta* isomer **9** of **4** still inactivated the L1210/FR8 enzyme but not the Walker 256 enzyme. The other seven analogs showed either poor or no irreversible inhibition of the two tumor enzymes.

We have previously reported[3] on the irreversible inhibition of the dihydrofolic reductases from three species by six dihydro-*s*-triazines of types **1** and **2**, where $n = 0$ 2. The enzyme from pigeon liver was irreversibly inhibited by all six compounds, but at varying rates. The enzymes from Walker 256 rat tumor and L1210/FR8 mouse leukemia were irreversibly inhibited by only two of the six compounds, namely **2** with $n = 0$ or 2. Therefore a further study was initiated on nine structural variants of **1** and **2** to see which could still inactivate either of the two tumor enzymes. The results are the subject of this paper.



1, *meta* series
2, *para* series

The enzyme studies on the nine variants of the two active analogs, **3** and **4**, are collated in Table I. Current evidence[5] indicates (a) the diamino-*s*-triazine moiety of these inhibitors is complexed within the active site in the region normally complexing the pteridine moiety of the substrate, dihydrofolate;[6] (b) the 1-phenyl moiety attached to the triazine is complexed to a hydrophobic bonding region that is not part of, but is adjacent to, the active site;[7] and (c) the sulfonyl fluoride group of the inhibitor forms a covalent bond with an amino acid on the surface of the enzyme that is not part of the active site.[8] Since it is outside the active site that evolutionary changes in enzyme structure are more apt to have occurred,[8] species, and even tissue, differences in the ability of the sulfonyl fluoride to attack an amino acid such as serine should be observable. Some such species differences with compounds of type **1** and **2** have already been reported.[3] These concepts are further supported by comparison of the results with **3** and its position isomer **5**.

It should be borne in mind that the rate of inactivation of an enzyme by the active-site-directed mechanism is dependent upon the concentration of reversible enzyme inhibitor complex, EI, and is not directly dependent on the concentration of inhibitor.[9,10] Both **3** and its *meta* isomer (**5**) rapidly inactivate the dihydrofolic reductase from Walker 256 at the same concentration of reversible EI complex. In contrast, the *meta* isomer (**5**) fails to inactivate the L1210 enzyme while the *para* isomer (**3**) rapidly inactivates this enzyme; thus, the species difference between a rat tissue (Walker 256) and mouse tissue (L1210) is apparent.

The next higher homolog (**6**) of the *para* isomer (**3**) showed little differences in reversible binding to the Walker 256 or L1210 enzymes; however, irreversible inhibition was dramatically different in that **6** failed to inactivate the L1210 enzyme and was only weakly effective on the Walker 256 enzyme. The *meta* (**7**) and *ortho* (**8**) isomers of **6** showed little change in reversible inhibition of the two enzymes; neither **7** nor **8** was an irreversible inhibitor of either enzyme.

Two analogs of the second irreversible inhibitor **4** were also investigated. Although **4** could rapidly inactivate the Walker 256 enzyme,[3] it was a poor reversible inhibitor and required concentrations several magnitudes higher than **3** to give the same amount of EI complex; **4** was also a poor reversible inhibitor of the L1210 enzyme and, in addition, inactivated considerably less effectively than **3**. When the sulfonyl fluoride group of **4** was moved to the *meta* position, the resultant **9** still complexed reversibly to the two enzymes at about the same magnitude as **4**. Although **4** and **9** inactivated the L1210 enzyme with about the same ef-

(1) This work was generously supported by Grant CA-08697 from the National Cancer Institute, U. S. Public Health Service.

(2) For the previous paper of this series, see B. R. Baker and M. A. Johnson, *J. Heterocyclic Chem.*, in press.

(3) For the previous paper on the sulfonyl fluoride type of irreversible inhibitor of dihydrofolic reductase see B. R. Baker and G. J. Lourens, *J. Med. Chem.*, **10**, 1113 (1967), paper CV of this series.

(4) G. J. L. wishes to thank the Council for Scientific and Industrial Research, Republic of South Africa, for a tuition scholarship.

(5) For a review on the mode of binding of inhibitors to dihydrofolic reductase see B. R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibitors. The Organic Chemistry of the Enzymic Active-Site," John Wiley and Sons, Inc., New York, N. Y., 1967, Chapter X.

(6) B. R. Baker and R.-T. Ho, *J. Pharm. Sci.*, **55**, 170 (1966).

(7) See B. R. Baker, T. J. Schwan, J. Nevosad, and R.-T. Ho, *ibid.*, **55**, 285 (1966); (b) B. R. Baker and H. S. Shapiro, *ibid.*, **55**, 308 (1966).

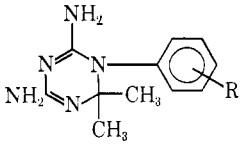
(8) See ref. 5, Chapter IX.

(9) B. R. Baker, W. W. Lee, and E. Tong, *J. Theoret. Biol.*, **3**, 158 (1962).

(10) For a discussion of the kinetics of irreversible enzyme inhibition see ref. 5, Chapter VIII.

TABLE I

INHIBITION[a] OF DIHYDROFOLIC REDUCTASES BY

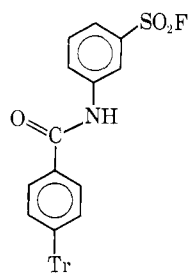


No.	R	Enzyme source[b]	Reversible: I_{50},[c] μM	Reversible: Estd $K_i \times 10^8$ M[d]	Irreversible[e]: Inhib concn, μM	Irreversible[e]: % EI[f]	Irreversible[e]: Time, min	Irreversible[e]: % inact
3[g]	*p*-$(CH_2)_2CONHC_6H_4SO_2F$-*p*	W256	0.020	0.0033	0.050	95	<1	90
		L1210	0.080	0.013	0.070	84	<2	84
4[g]	*p*-$CONHC_6H_4SO_2F$-*p*	W256	21	3.5	25	88	<1	100
		L1210	600	100	25	20	60	45
5	*p*-$(CH_2)_2CONHC_6H_4SO_2F$-*m*	W256	0.0080	0.0013	0.060	97	5	100
		L1210	0.044	0.0073	0.20	96	60	0
6	*p*-$(CH_2)_3CONHC_6H_4SO_2F$-*p*	W256	0.022	0.0037	0.11	97	60	22
		L1210	0.042	0.0070	0.21	97	60	0
7	*p*-$(CH_2)_3CONHC_6H_4SO_2F$-*m*	W256	0.012	0.0020	0.060	97	60	0
		L1210	0.060	0.010	0.30	97	60	0
8	*p*-$(CH_2)_3CONHC_6H_4SO_2F$-*o*	W256	0.016	0.0027	0.078	97	60	0
		L1210	0.060	0.010	0.30	97	60	0
9	*p*-$CONHC_6H_4SO_2F$-*m*	W256	21	3.5	25	87	120	0
		L1210	110	18	25	62	60	44
10	*m*-$CONHC_6H_4SO_2F$-*m*	W256	0.25	0.042	1.4	97	120	0
		L1210	0.63	0.11	4.0	97	60	0
11	*p*-$CH_2CONHC_6H_4SO_2F$-*m*	W256	0.0087	0.0014	0.024	94	120	0
		L1210	0.031	0.0052	0.15	97	60	0
12	*m*-SO_2F	W256	0.054	0.0090	0.25	97	120	0
		L1210	0.14	0.023	0.70	97	60	0
13	*m*-$CH_2NHCONHC_6H_4SO_2F$-*m*	Pigeon liver	0.063	0.011	0.060	86	<2	>90
		W256	0.024	0.0040	0.12	97	60	0
		L1210	0.066	0.011	0.33	97	60	0
14[g]	*m*-$CH_2CH_2CONHC_6H_4SO_2F$-*p*	Pigeon liver	0.10	0.017	0.21	90	8[h]	50
		W256	0.064	0.011	0.32	97	120	0
		L1210	0.078	0.013	0.40	97	60	0

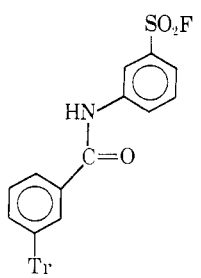
[a] The technical assistance of Barbara Baine, Jean Reeder, and Diane Shea with these assays is acknowledged. [b] W256 = Walker 256 rat tumor, L1210 = L1210/FR8 mouse leukemia. [c] I_{50} = μM inhibitor concentration necessary for 50% inhibition of the enzyme in the presence of 6 μM dihydrofolate and 30 μM TPNH at pH 7.4 by the methods previously described.[3] [d] Calculated from $K_i = K_m(I_{50}/[S])$ where $K_m \simeq 1 \times 10^{-6}$ M and $[S] = 6 \times 10^{-6}$ M; this equation is valid where $K_m > 4[S]$.[5] [e] The specified concentration of inhibitor was incubated with the enzyme in the presence of 60 μM TPNH at pH 7.4 and 37°, then the remaining enzyme was assayed as previously described.[3] [f] Percent of enzyme reversibly complexed as calculated from $[EI] = [E_t]/(1 + K_i/[I])$.[8,9] [g] Data from ref 3. [h] Half-time of inactivation.

fectiveness, **9** failed to inactivate the Walker 256 enzyme.

There are four possible ground-state conformations for **9**, of which presumably only one can cause inactivation of the L1210 enzyme. Similarly, there are four ground-state conformations for its analog **10**; of these four conformations each for **9** and **10**, there is one conformation of each that positions the attacking sulfonyl fluoride in a nearly identical position. Therefore, **10** was synthesized for enzymic evaluation. Since **10** failed to inactivate the L1210 enzyme, it does not appear likely that **9** has conformation **9a** when it inactivates the enzyme but presumably has one of the other



9a



10a

Tr = triazine system

three ground-state conformations during inactivation. This result was unfortunate from another standpoint; **10** is a much better reversible inhibitor than **9** and requires less than $^1/_{100}$ as much inhibitor to give the same amount of reversible enzyme–inhibitor complex.

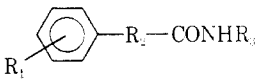
Along these same lines, **11** was synthesized as an analog of **4** that should show better reversible inhibition and might attack the same amino acid. Although **11** was a much better reversible inhibitor than **4**, it failed to inactivate either enzyme.

For further supporting evidence that (a) **3–5** inactivate by the active-site-directed mechanism[3] and (b) that the 1-phenyl group attached to the *s*-triazine is complexed in a hydrophobic region, **12** was synthesized for enzymic evaluation. As anticipated, **12** failed to inactivate either enzyme showing that (a) **4** could not have inactivated the enzymes by a bimolecular mechanism, and (b) there was not a polar amino acid in this vicinity capable of reacting with the sulfonyl fluoride of **12**.

The last compound in this series was **13** which can be considered an analog of **14**; the latter compound was previously shown[3] to inactivate the pigeon liver enzyme, but not the enzymes from Walker 256 or L1210. A similar pattern was again observed where only the

Table II

Physical Properties[a] of

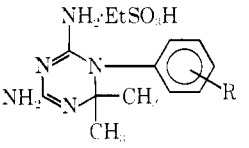


R_1	R_2	R_3	Method[b]	% yield	Mp, °C	Formula[c]
p-NO_2	...	$C_6H_4SO_2F$-*m*	A	62[d]	218–221	$C_{13}H_9FN_2O_5S$
p-NH_2	...	$C_6H_4SO_2F$-*m*	B	74[e]	163–164	$C_{13}H_{11}FN_2O_3S$
m-NO_2	...	$C_6H_4SO_2F$-*m*	A	59[f]	170–171	$C_{13}H_9FN_2O_5S$
m-NH_2	...	$C_6H_4SO_2F$-*m*	B	43[g]	148–149	$C_{13}H_{11}FN_2O_3S$
p-NO_2[h]	CH_2	$C_6H_4SO_2F$-*m*	A	72[d]	150–151	$C_{14}H_{11}FN_2O_5S$
p-NO_2[h]	CH=CH	$C_6H_4SO_2F$-*m*	A	73[d]	dec pt >270	$C_{15}H_{11}FN_2O_5S$
p-NO_2	$(CH_2)_2$	$C_6H_4SO_2F$-*p*	A	55[f]	149–151	$C_{16}H_{15}FN_2O_5S$
p-NH_2	$(CH_2)_3$	$C_6H_4SO_2F$-*p*	B	83[e]	130–132	$C_{16}H_{17}FN_2O_3S$
p-NO_2[h]	$(CH_2)_3$	$C_6H_4SO_2F$-*m*	A	68[e]	103–104	$C_{16}H_{15}FN_2O_5S$
p-NO_2[h]	$(CH_2)_3$	$C_6H_4SO_2F$-*o*	A	45[f]	125–126	$C_{16}H_{15}FN_2O_5S$

[a] Each compound moved as a single spot on tlc on Brinkmann silica gel GF. [b] A, reaction of acid chloride with amine[3] in refluxing toluene; B, catalytic reduction with Raney Ni catalyst.[3] [c] All compounds were analyzed for C, H, N. The analytical results for these elements were within ±0.3% of the theoretical values. [d] Recrystallized from 2-methoxyethanol. [e] Recrystallized from EtOH–H_2O. [f] Recrystallized from EtOH. [g] Recrystallized from *i*-PrOH. [h] The corresponding amine prepared by method B was not crystallized, but was pure on tlc; it was converted directly to the corresponding dihydro-*s*-triazine in Table III.

Table III

Physical Properties[a] of



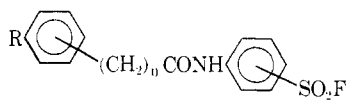
No.	R	% yield	Mp, °C dec	Formula[b]
5	*p*-$(CH_2)_2CONHC_6H_4SO_2F$-*m*	65[c,d]	207–208	$C_{20}H_{23}FN_6O_3S \cdot C_2H_5SO_3H$
6	*p*-$(CH_2)_3CONHC_6H_4SO_2F$-*p*	76[d]	208–209	$C_{21}H_{25}FN_6O_3S \cdot C_2H_5SO_3H$
7	*p*-$(CH_2)_3CONHC_6H_4SO_2F$-*m*	34[c,d]	dec pt >125	$C_{21}H_{25}FN_6O_3S \cdot HCl$[e,f]
8	*p*-$(CH_2)_3CONHC_6H_4SO_2F$-*o*	44[c,d]	186–187	$C_{21}H_{25}FN_6O_3S \cdot C_2H_5SO_3H$
9	*p*-$CONHC_6H_4SO_2F$-*m*	72[d]	212–213	$C_{18}H_{19}FN_6O_3S \cdot C_2H_5SO_3H$
10	*m*-$CONHC_6H_4SO_2F$-*m*	67[d]	200–201	$C_{18}H_{19}FN_6O_3S \cdot C_2H_5SO_3H$
11	*p*-$CH_2CONHC_6H_4SO_2F$-*m*	56[c,g]	163–164	$C_{19}H_{21}FN_6O_3S \cdot C_2H_5SO_3H$
12	*m*-SO_2F	39[c,d]	192–193	$C_{11}H_{14}FN_5O_2S \cdot C_2H_5SO_3H$
13	*m*-$CH_2NHCONHC_6H_4SO_2F$-*m*	76[h,i]	154–155	$C_{19}H_{22}FN_7O_3S \cdot C_2H_5SO_3H$

[a] All compounds except **13** were prepared by condensation of the appropriate arylamine from Table II with cyanoguanidine and acetone.[3,10] [b] All compounds were analyzed for C, H, N. The analytical results for these elements were within ±0.3% of the theoretical values. [c] Over-all yield from nitro compound without isolation of the amine. [d] Recrystallized from aqueous *i*-PrOH. [e] Hydrochloride; the ethanesulfonate could not be crystallized. [f] Calculated for 0.5 *i*-PrOH. [g] Recrystallized from EtOH. [h] Recrystallized from EtOH–petroleum ether (bp 30–60°). [i] See Experimental Section.

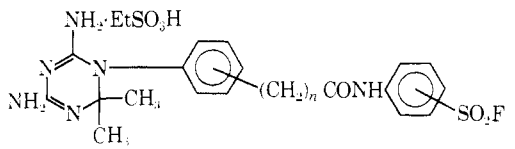
pigeon liver enzyme was inactivated; however, **13** inactivated the pigeon liver enzyme more rapidly than did **14**.

Of the fifteen dihydro-*s*-triazines so far evaluated, only **3** was a fast irreversible inhibitor of the L1210 enzyme, but both **3** and **5** were fast irreversible inhibitor of the Walker 256 enzyme. Thus, the optimum distance between the inside and outside phenyl is apparently four atoms. Whether other four-atom bridges can be used in **3** so that it still gives an irreversible inhibitor is the subject of a paper to follow; whether such changes in **5** are feasible is under investigation.

Chemistry.—Compounds **5**–**11**, which can be generalized by structure **17**, were synthesized in the same manner used for **3** and **4**.[3] The appropriate aminobenzenesulfonyl fluoride was acylated with the appropriate nitrophenylacyl chloride to give **15** (Table II); in the case of **5**, *p*-nitrocinnamoyl chloride was employed. The nitro group was reduced catalytically with a Raney nickel catalyst and the resultant amine



15, R = NO_2
16, R = NH_2



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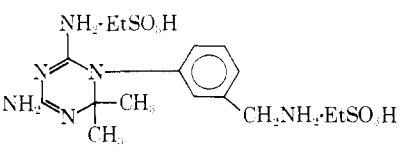

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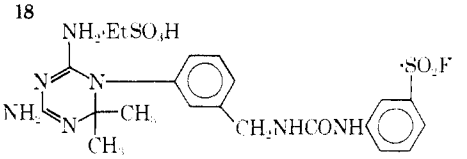

13

(**16**) was converted to the dihydro-*s*-triazine by the three-component method of Modest.[11]

Reaction of *m*-fluorosulfonylphenyl isocyanate with **18**[12] in the presence of 1 equiv of triethylamine afforded **13**. The last compound, **12**, was synthesized from *m*-aminobenzenesulfonyl fluoride, cyanoguanidine, and acetone according to the general method of Modest.[11]

Experimental Section[13]

4,6-Diamino-1,2-dihydro-2,2-dimethyl-1-[*m*-(*m*-fluorosulfonylphenylureidomethyl)phenyl]-*s*-triazine Ethanesulfonate (13).—To a mixture of 117 mg (0.25 mmole) of **18**,[12] 0.2 ml of DMF, and 0.13 ml of 2 m*M* Et_3N in DMF stirred in an ice bath was added 75 mg (0.38 mmole) of *m*-fluorosulfonylphenyl isocyanate (Aldrich) in 0.10 ml of DMF. Within 5 min the clear solution began to deposit white crystals. After 15 min, the mixture was diluted with 1 ml of reagent Me_2CO, then stirred at ambient temperature for 40 min. The product was collected on a filter and washed with Me_2CO. Recrystallization from EtOH–petroleum ether (bp 30–60°) gave 105 mg (76%) of white crystals: mp 154–155°; λ_{max}^{EtOH} 249, 299 (weak) mμ. See Table III for additional data.

(11) E. J. Modest, *J. Org. Chem.*, **21**, 1 (1956).

(12) The synthesis of this compound in two steps from *m*-aminobenzonitrile has been previously described by B. R. Baker and G. J. Lourens, *J. Med. Chem.*, **11**, 26 (1968), paper CIX of this series.

(13) Melting points were taken in capillary tubes on a Mel-Temp block and are uncorrected. All analytical samples gave ir and uv spectra compatible with their assigned structures.

Lipid-Soluble Derivatives of 6-Mercaptopurine[1]

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Several S,9-dialkyl derivatives of 6-mercaptopurine designed for lipid solubility have been synthesized and evaluated against Adenocarcinoma 755 implanted both subcutaneously and intercerebrally and against leukemia L1210 implanted intraperitoneally and intracerebrally to assess their ability to cross the blood–brain barrier. One compound 6-(cyclopentylthio)-9-ethylpurine appears to be more effective than 6-mercaptopurine against the intracerebral diseases.

Many of the most potent anticancer agents that are in use today, including 6-mercaptopurine, are ineffective against leukemia L1210 implanted intracerebrally in mice.[2] Since we had previously found that certain 9-alkyl derivatives of 6-mercaptopurine[3] are highly active against Adenocarcinoma 755 implanted intraperitoneally in mice,[4] it seemed reasonable to synthesize and evaluate a series of S,9-disubstituted derivatives of 6-mercaptopurine, designed for lipid solubility, that might penetrate the blood–brain barrier[5] better than 6-mercaptopurine itself. To this end the anions of 9-ethylpurine-6(1H)-thione and 9-butylpurine-6(1H)-thione were alkylated in N,N-dimethylformamide in the usual manner[6] to give the desired S-alkyl derivatives **1–6** (see Experimental Section). The synthesis[3] and evaluation against Adenocarcinoma 755 implanted subcutaneously[4] of 9-ethyl-6-methylthiopurine was reported previously.

Results and Discussion

All of the S-alkyl compounds were effective in inhibiting the growth of Adenocarcinoma 755 implanted subcutaneously, although the octylthio compounds (**3** and **6**) were significantly less effective than the others (Table I). As judged by therapeutic index the 9-ethyl compounds (**1–3**) were more effective than the butyl compounds (**4–6**) (Table III). All the compounds prolonged the life of mice implanted intracerebrally with Ad755 cells, although the activity of the octylthio compounds and of 9-butyl-6-methylthiopurine was minimal. Again the 9-ethyl compounds appear to be more effective than 9-butyl compounds (Table II). None of the compounds, however, were more effective than 6-mercaptopurine (6-MP) (Table III), and only two, **1** and **2**, were as effective. These results indicate that 6-mercaptopurine itself can cross the blood–brain barrier in sufficient quantity to profoundly affect the growth of a sensitive tumor,[4] Ad755. Although it is likely that the S,9-dialkyl derivatives which are quite soluble in organic solvents, cross the "barrier" more easily than 6-mercaptopurine, most of them are less effective than 6-mercaptopurine against the intracerebral disease, presumably because they are inherently less effective in inhibiting the growth of Ad755, as can be seen from their therapeutic index against the subcutaneous tumor where entry into the brain is not involved (Table III).

In order to determine if the two highly active S,9-dialkyl derivatives **1** and **2** were active against less sensitive cancer cells implanted intracerebrally, they were evaluated against L1210 leukemia cells implanted both intraperitoneally and intracerebrally (Table IV). **1** was only slightly effective against the intraperitoneal disease and **2** was more effective than **1** but less effective than 6-MP, which in repeated runs has increased the lifespan of intraperitoneal-leukemic mice 70–80% on the average. On the other hand, 6-(cyclopentylthio)-9-ethylpurine (**2**) increased by 64–69% the life-

(1) This work was supported by funds from the C. F. Kettering Foundation and the Cancer Chemotherapy National Service Center, National Cancer Institute, National Institutes of Health, Contract No. PH43-64-51.

(2) H. E. Skipper, F. M. Schabel, Jr., M. W. Trader, and J. R. Thomson, *Cancer Res.*, **21**, 1154 (1961).

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