under autogeneous pressure. The solid (3.6 g, 88%, mp 202–205°) was recrystd from $Me_2CO$–petr ether (bp 60–110°) to yield 2.0 g (49%) of XLVI, mp 204–206°. *Anal.* ($C_{12}H_{11}ClN_2O_2S$): C, H, N.

An attempt to shorten the reaction time for the prepn of XLVI at 270–300° (19 hr) resulted in 2.6 g (56%) of XLV, mp 216–219° (from $Me_2CO$). *Anal.* ($C_{12}H_9Cl_2NO_2S$): N. Acetylation of XLV gave XLIV, mp 214–215° (no mp depression with authentic XLIV).

**Acknowledgment.**—Miss P. M. Thomas assisted us in the preparation of a few of the compounds. The analyses were carried out by Pennwalt's Analytical Department.

# Inhibition of Electron Transport by Substituted Salicyl-*N*-(*n*-octadecyl)amides†

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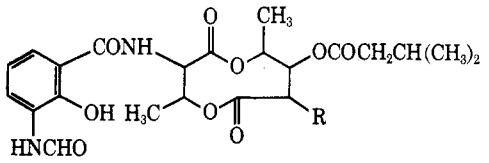
Several new analogs of antimycin A have been synthesized which were inhibitory toward submitochondrial succinate oxidase, NADH-oxidase, and succinate–cytochrome *c* reductase. The degree of inhibition was generally greatest when the substituted salicyl-*N*-(*n*-octadecyl)amides were preincubated with the electron transport particles. Inhibitions greater than 90% were observed with 5-nitro-, 5-formamido-, and 3,5-dinitrosalicyl-*N*-(*n*-octadecyl)amides. Salicyl-*N*-(*n*-octadecyl)amide was not inhibitory. The analog inhibitory site was similar to the antimycin A site as determined by an increased reduced cytochrome *b* (562 nm) peak and a decreased reduced cytochrome *c* (550 nm) peak in the inhibited difference spectra. The results demonstrated that a formamido substituent ortho to the phenolic OH of antimycin A is not required for inhibitory activity provided there is either a formamido or nitro group para to OH.

The characteristic inhibitory activity of antimycin A(I) on the electron transport system of higher animals is associated with the substituted aromatic moiety[1,2] while the remaining dilactone portion seeminly provides a required lipophilicity. Dickie, *et al.*,[2] reported that 3-formamidosalicyl-*N*-(*n*-octadecyl)amide inhibited the reduction of cytochrome *c* in a manner analogous to antimycin.




I

antimycin A

The observation that an analog having an $NO_2$ group substituted for the formamido function retained a diminished inhibitory activity,[2] suggested that an acylated amino group[3] was functionally replaceable. The phenolic OH of antimycin is strongly acidic[4] and required for inhibitory activity.[5] If the function of the NHCHO and $NO_2$ groups ortho to the phenolic OH is merely to enhance acidity, then analogs containing combinations of ortho and para substituents should also show inhibitory properties. The synthesis of several previously unreported substituted salicyl-*N*-(*n*-octadecyl)amides and their inhibitory capacity toward submitochondrial electron transport are reported in this paper.

## Experimental Section‡

**Substituted Salicyl-*N*-(*n*-octadecyl)amides.**—These compds were prepd from the appropriately substituted salicylic acid by a modification of the method of Dickie, *et al.*[2] As an example, the prepn of 3-formamido-5-nitrosalicyl-*N*-(*n*-octadecyl)amide is described in detail.

To 30 ml of 88% $HCO_2H$ contg 2.0 g of sodium formate was added 2.5 g (12 mmoles) of 3-amino-5-nitrosalicylic acid monohydrate, mp 227–228°, prepd by the method of Meldola, *et al.*[6] After heating in a water bath for 1 hr, 30 ml of $H_2O$ was added and the heating contd for 5 min. Upon cooling to room temp the formylated product pptd and was collected by filtration to yield 1.2 g (5.0 mmoles). This product was immediately dissolved in 25 ml of THF and 2.86 g (10.5 mmoles) of *n*-octadecylamine was dissolved with stirring. A THF soln (75 ml) contg 1.2 g (5.8 mmoles) of DCI was slowly added, and the soln was stirred at room temp for 20 hr. The reaction mixt was acidified with 25 ml of glacial AcOH followed by addn of 12 ml of $H_2O$ to ppt dicyclohexylurea which was filtered and discarded. The filtrate was evapd in a rotary evaporator, and the residue was crystd from AcOH–$H_2O$. Recrystn from EtOH–$H_2O$ yielded 1.55 g (3.2 mmoles, 27%) of a bright yellow product, mp 115.5–116.5°. *Anal.* ($C_{26}H_{43}N_3O_5 \cdot H_2O$) C, H, N.

**Prepn of Mitochondria and Submitochondrial Particles.**—Beef heart mitochondria were prepd by the methods described by Crane, *et al.*[7] These prepns remained stable for several weeks when stored at −20°. Submitochondrial particles were prepd by a modification of the method of Urban, *et al.*,[8] Thawed mitochondrial prepns were adjusted to pH 8.0 with 1 *N* NaOH followed by sonication with a Bronwill Biosonik Model 11 at a probe intensity of 70 for 2 min at 4°. The sonicate was centrifuged at 40,000*g* for 10 min, and the supernatant was decanted and saved. The pellet was suspended in a pH 7.5, 0.01 *M* Tris–succinate buffer soln which contd 0.25 *M* mannitol and 0.2 m*M* EDTA. The suspension was adjusted to pH 8.0, resonicated, and centrifuged at 40,000*g*. The 2 supernatants were combined and centrifuged at 70,000*g* for 30 min. The pellet was washed 3 times with 0.05 *M* phosphate buffer (pH 7.5) and suspended in the Tris–mannitol soln at a protein concn of 10 mg/ml. Protein concns were detd by the method of Folin and Ciocalteau.[9]

**Assay for Inhibition of NADH and Succinate Oxidase.**—

† Supported in part by Grant No. AI 08621 from the National Institutes of Health.

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‡ Where analyses are indicated only by symbols of the elements, anal. results obtained for those elements were within ±0.4% of the theor values.

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NADH and succinate oxidase were assayed at 32° with a YSI Model 53 oxygen monitor equipped with a Clark electrode (Yellow Springs Instrument Company, Yellow Springs, Ohio). The assay medium contd 85 μmoles of $PO_4$, pH 7.5; 75 μmoles of KCl; 5 μmoles of $MgCl_2$; 2.5 mg of submitochondrial particle protein; and either 45 μmoles of potassium succinate or 3 μmoles of NADH (Sigma, Type III, disodium salt) in a final vol of 3.0 ml.

The inhibitors were dissolved in EtOH and 0.01 ml of the soln was added either directly to the assay system or preincubated with the enzyme for 30 min prior to assay.

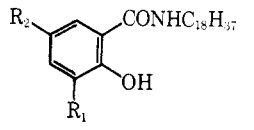
**Assay for Inhibition of Succinate–Cytochrome *c* Reductase.**—Spectrophotometric assay of succinate–cytochrome *c* reductase activity was detd by the methods described by Kaniuga, *et al.*[10] The final assay mixt contd 150 μmoles of $PO_4$, pH 7.4; 60 μmoles of potassium succinate; 0.78 mg of cytochrome *c* (Sigma, Type III); 3.0 μmoles of KCN; 5.0 μmoles of EDTA; 10 mg of sodium deoxycholate; and 1 mg of submitochondrial protein in a final vol of 3.0 ml.

## Discussion

The preparation of the octadecylamides of the various substituted salicylic acids (Table I) invariably produced

TABLE I

SALICYL-*N*-(*n*-OCTADECYL)AMIDES




| Compd[a] | Mp, °C[d] | Yield, % | Formula | Analysis |
|---|---|---|---|---|
| $R_1 = H; R_2 = NO_2$ (I) | 99.5–100 | 61 | $C_{25}H_{42}N_2O_4$ | C, H, N |
| $R_1 = H; R_2 = NHCHO$ (II) | 119.5 | 58 | $C_{26}H_{44}N_2O_3$ | C, H, N |
| $R_1 = R_2 = NO_2$[b] (III) | 179–180 | 53 | $C_{48}H_{80}N_4O_6$ | C, H, N |
| $R_1 = NHCHO; R_2 = NO_2$[c] (IV) | 115.5–116.5 | 27 | $C_{26}H_{45}N_3O_6$ | C, H, N |
| $R_1 = R_2 = H$ (V) | 87–87.5 | 30 | $C_{25}H_{43}NO_2$ | H, N; C[e] |

[a] Recrystd from EtOH–$H_2O$. [b] Isolated as the octadecyl amine salt. [c] Isolated with 1 $H_2O$ of solvation. [d] All melting points were observed on a Thomas-Hoover Uni-Melt capillary apparatus and are cor. [e] C: calcd, 77.20; found, 77.91.

an octadecylamine salt with the acidic phenol, a result which has been previously reported.[2] In the case of the 2,4-dinitrosalicyl analog, the octadecylamine salt could not be dissociated by crystallization from acidic solvent systems as previously described.[2] The possible formation of a Meisenheimer type salt[11] was supported by the low-field absorption of the aromatic protons, δ −8.57 and −8.79.[12] Assay of this salt was impeded by its low solubility in the test systems.

The relative inhibitory activities of the substituted salicyl-*N*-(*n*-octadecyl) analogs were compared in all test systems with the activities of the previously reported 3-nitro- (VI) and 3-formamidosalicyl-*N*-(*n*-octadecyl)amides (VII). (Tables II and III). The latter compound had been found to be slightly less active than antimycin A in a reduced coenzyme Q–cytochrome *c* reductase system.[2]

The analogs which were inhibitory showed an accumulation of reduced cytochrome *b* and oxidized cytochrome *c* as observed by difference spectroscopy at 563 and 550 mμ[13] which is consistent with the previously reported site of antimycin inhibition.[14]

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(12) R. Foster and C. A. Fyfe, *Tetrahedron*, **21**, 3363 (1965).
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TABLE II

INHIBITION OF SUBMITOCHONDRIAL SUCCINOXIDASE BY SUBSTITUTED SALICYL-*N*-(*n*-OCTADECYL)AMIDES

| | % inhibition | |
|---|---|---|
| Compd[a] | Preincubation[c] | Direct addition[d] |
| Control | 0 | 0 |
| I | 81 | 81 |
| II | 8 | 0 |
| III[b] | 52 | 0 |
| IV | 59 | 64 |
| V | 27 | 0 |
| VI | 75 | 82 |
| VII | 75 | 81 |

[a] Inhibitor concns were 46 nmole/mg of protein. [b] Octadecyl amine salt. [c] Preincubation with submitochondrial particles for 30 min at 4°. [d] Inhibitor added prior to the addn of succinate.

TABLE III

INHIBITION OF SUBMITOCHONDRIAL NADH-OXIDASE AND SUCCINATE–CYTOCHROME *c* REDUCTASE BY SUBSTITUTED SALICYL-*N*-(*n*-OCTADECYL)AMIDES[a]

| | % inhibition | |
|---|---|---|
| Compd[b] | NADH-oxidase | Succinate–cytochrome *c* reductase[d] |
| I | 98 | 71 |
| II | 30 | 57[e] |
| III[c] | 38 | 32[f] |
| IV | 85 | 77 |
| V | 25 | 0 |
| VI | 97 | 73 |
| VII | 97 | 85 |

[a] Inhibitor was added after the reaction had been started. Inhibn was measured when a new rate of $O_2$ uptake or cytochrome *c* reduction had been established. [b] Inhibitor concns were 115 nmoles/mg of protein for the cytochrome *c* reductase and 46 nmoles/mg of protein for the NADH-oxidase assays. [c] Octadecyl amine salt. [d] Detd by the change in rate of reduction of cytochrome *c* as measured at 550 mμ in a Cary Model 15 spectrophotometer. [e] Assayed at the max solubility of 23 nmoles/mg of protein. [f] Assayed at the max solubility of 2.6 nmoles/mg of protein.

The unsubstituted salicylamide analog was essentially inactive under all of the assay conditions. This suggests that a third aromatic substituent is required. However, it is now apparent that the substituent need not be in the 3 position since the 5-nitrosalicylamide was as active as either the 3-nitro- or 3-formamidosalicyl-*N*-(*n*-octadecyl)amides. The results in Tables II and III suggested that 5-formamidosalicyl-*N*-(*n*-octadecyl)amide was less active than the 5-nitro analog, however, solubility limitations in these assay systems make this conclusion doubtful since the 5-formamido analog exhibited reasonable activity at a 5-fold lower concentration than the other analogs in the succinate–cytochrome *c* reductase system (Table III).

The relative inactivity of the octadecylamine salt of 3,5-dinitrosalicyl-*N*-(*n*-octadecyl)amide (Tables II and III) might be ascribed to either a decreased lipophilicity or a limited dissociation to the free phenol required for inhibitory activity. The inability to dissociate this salt by chemical methods is evidence for the latter explanation.

The results clearly demonstrated that a formamido substituent ortho to the phenolic hydroxyl of antimycin A is not required for inhibitory activity provided there is either a formamido or nitro group para to the OH.