(10)]. The 4.6-kb Eco RI fragments carrying the *lac*-promoted out-of-frame construct and in-frame fusion were then inserted into pEW49 [a derivative of pEW27 (21) with a single Eco RI site], and the resulting plasmids (pEW80 and pEW76, respectively) mobilized to WAg17 (21) as described in the legend to Fig. 1. Exconjugants containing Ti plasmid–borne tandem arrays of the integrative plasmids were selected, and their structure was confirmed by restriction digestion and DNA blot analysis (18). *Vir D* sequences were bases 100 to 1420 of GenBank access code M14762.

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21. pEW27 is a 6.7-kb derivative of pBR322 [F. Bolivar *et al.*, *Gene* **2**, 95 (1977)] that confers tetracycline resistance and carries the left border of Ti plasmid pTiT37 as a 1.4-kb Eco RI fragment [N. S. Yadav, K. Postle, R. K. Saiki, M. F. Thomashow, M.-D. Chilton, *Nature* **287**, 458 (1980)]. WAg17 was constructed from *A. tumefaciens* strain A208 [D. Sciaky, A. L. Montoya, M.-D. Chilton, *Plasmid* **1**, 238 (1978)] by the method of A. J. Matzke and M.-D. Chilton [*J. Mol. Appl. Genet.* **1**, 39 (1981)]. WAg17 harbors a deleted derivative of pTiT37, having a 4.6-kb T region and sharing homology with pBR322 and pEW27. Details of the constructions are available upon request. *Agrobacterium tumefaciens* exconjugants resistant to ≥12 μg of tetracycline per milliliter always harbor Ti plasmids bearing multiple tandem insertions of the small donor plasmid, since one copy of the tetracycline resistance gene is insufficient for resistance to this level of the drug (18).
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A Pituitary *N*-Acetylgalactosamine Transferase That Specifically Recognizes Glycoprotein Hormones

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The glycoprotein hormones lutropin (LH) and follitropin (FSH), which have common α-subunits but hormone-specific β-subunits, are both synthesized in the gonadotroph. However, they bear Asn-linked oligosaccharides that differ in structure. Those on LH terminate with the sequence SO_4-4GalNAcβ1→4GlcNAcβ1→2Manα, whereas those on FSH terminate with the sequence sialic acidα–Galβ1→4GlcNAcβ1→2Manα. A GalNAc-transferase was identified in bovine pituitary membranes that recognizes features of the α-subunit peptide and adds GalNAc to its oligosaccharides with an apparent Michaelis constant of 25 micromolar. The different patterns of glycosylation for LH and FSH indicate that access to the protein recognition marker on the α-subunit is modulated by the associated β-subunit. The tightly regulated synthesis of sulfated and sialylated oligosaccharides on the pituitary glycoprotein hormones suggests these oligosaccharides have an important biological role.

THE GLYCOPROTEIN HORMONES ARE a family of four closely related dimeric proteins with common α-subunits and hormone-specific β-subunits. Even though the amino acid sequences of the β-subunits of LH (lutropin), FSH (follitropin), TSH (thyrotropin), and hCG (human chorionic gonadotropin) are closely related, the Asn-linked oligosaccharides present on their α- and β-subunits differ in structure (*1–3*). Two of the hormones, LH and FSH, are synthesized within the same cell in the pituitary, the gonadotroph (*1, 4, 5*). LH bears Asn-linked oligosaccharides terminating with the sequence SO_4-4GalNAcβ1→4GlcNAcβ1→2Manα (Fig. 1), whereas FSH and other pituitary glycoproteins contain Asn-linked oligosaccharides terminating with the sequence sialic acidα–Galβ1→4GlcNAcβ1→2Manα (Fig. 1). Sulfated oligosaccharides such as S-2 have to date only been found on certain of the glycoprotein hormones (*1, 6, 7*). The presence of different oligosaccharide structures on LH and FSH indicates that glycosylation of these hormones is tightly regulated.

The steps leading to the synthesis of sulfated and sialylated oligosaccharides are illustrated in Fig. 1. The synthetic intermediates enclosed by the broken lines are precursors for both sialylated and sulfated oligosaccharides. Thus, either Gal or GalNAc can be added to the product of step 2. For FSH and the vast majority of other pituitary glycoproteins, Gal is added to the product of step 2, followed by sialic acid (steps 5 and 6). In contrast, GalNAc and sulfate are sequentially added to the identical synthetic intermediates on LH (steps 3 and 4). Since the oligosaccharide intermediates present on the glycoprotein hormones and other pituitary glycoproteins are identical, addition of GalNAc rather than Gal must involve a GalNAc-transferase that recognizes features of the α- and β-subunits of LH. Uncombined (free) α-subunits synthesized by bovine pituitaries, like LH, bear sulfated rather than sialylated oligosaccharides (*8*). This suggested that a recognition marker present on the α-subunit promotes the synthesis of sulfated oligosaccharides and that access to this recognition marker is modulated by the associated β-subunit, resulting in differential glycosylation of LH and FSH (*9*). We have identified a GalNAc-transferase in bovine pituitary membranes that displays specificity for the α-subunit of glycoprotein hormones and propose that this activity accounts for the synthesis of sulfated oligosaccharides on glycoprotein hormones and uncombined α-subunits.

The hCG α-subunit (hCGα) (*10, 11*), human transferrin, human α_1-antitrypsin, human J-chain (*12*), and bovine immunoglobulin G (IgG) (*13*) bear sialylated dibranched complex type oligosaccharides. Glycoproteins and glycopeptides bearing $GlcNAc_2Man_3GlcNAc_2$ (product of step 2 in Fig. 1) were prepared by enzymatic removal of sialic acid and Gal, and compared as substrates for GalNAc- and Gal-transferase activities present in crude bovine pituitary membrane preparations (Fig. 2 and Table 1). [^{3}H]Gal was transferred from uridine diphosphate (UDP)–Gal to agalacto-oligosaccharides on each of these substrates with apparent Michaelis constant (K_m) values between 160 and 900 μ*M*. The catalytic efficiency (V_{max}/K_m) was similar for transfer of Gal to each of these substrates (Table 1). The differences in the apparent K_m values obtained for addition of Gal to these substrates may reflect the presence of two oligosaccharide moieties on transferrin, α_1-antitrypsin, and hCGα as compared to a single oligosaccharide on J-chain and the glycopeptide.

Pituitary membranes also contained an enzyme activity that transferred [^{3}H]GalNAc to agalacto-oligosaccharides. When transfer of GalNAc and Gal were compared at similar substrate concentrations (Fig. 2), the GalNAc-transferase displayed a marked preference for hCGα. Although it was possible to detect transfer of GalNAc to each of the substrates examined, the apparent K_m values obtained were 16- to 60-fold as great as the apparent K_m for transfer of GalNAc to hCGα. The catalytic efficiency for transfer

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Table 1. Comparison of pituitary *N*-acetylgalactosamine- and galactose-transferases. The activities of GalNAc-transferase and Gal-transferase were measured as described in Fig. 2 with membranes containing 367 μU of GalNAc-transferase per milliliter (1 unit = 1 μmol transferred per minute at 37°C at a saturating concentration of agalacto hCGα).

Substrate	GalNAc-transferase			Gal-transferase		
	Apparent K_m (μ*M*)*	Apparent V_{max} (pmol hour^{-1} μg membranes^{-1})	Catalytic efficiency (V_{max}/K_m)†	Apparent K_m (μ*M*)*	Apparent V_{max} (pmol hour^{-1} μg membranes^{-1})	Catalytic efficiency (V_{max}/K_m)†
Glycopeptide	1000	1.1	3.8	900	27.5	1.2
J-chain	1500	0.8	1.9	470	8.5	0.7
Transferrin	900	0.2	1.0	430	11.0	1.0
α1-Antitrypsin	440	0.1	0.9	190	8.5	1.7
hCGα	25	1.1	165.8	160	6.0	1.4

*Calculated per mole of protein or glycopeptide. †Relative to human transferrin, which was set at 1.0. The 95% confidence limits for all catalytic efficiencies fell within ±6% of the determined values.

of GalNAc to hCGα was 87- to 166-fold as great as the values for transfer to the other three glycoproteins tested and 44-fold as great as the value obtained with the glycopeptide (Table 1). Therefore, the agalacto-oligosaccharide is a markedly better substrate for the GalNAc-transferase when attached to the hormone α-subunit than when it is attached either to a small peptide or to other glycoproteins.

The GalNAc- and Gal-transferase products were characterized to establish that GalNAc and Gal, respectively, had been incorporated into hCGα oligosaccharides in the appropriate linkage and location. [^{3}H]Gal and [^{3}H]GalNAc were the only monosaccharides detected following acid hydrolysis and were quantitatively released from hCGα by *N*-glycanase digestion (*14*), indicating that GalNAc and Gal were incorporated exclusively into Asn-linked oligosaccharides. The *N*-glycanase–released oligosaccharides comigrated with authentic oligosaccharide standards during ion-suppression, amine adsorption high-performance liquid chromatography, which fractionates oligosaccharides on the basis of size (*15*). [^{3}H]GalNAc was released by jack bean, but not diplococcal, β-*N*-acetyl-hexosaminidase. In addition, during lectin affinity chromatography the GalNAc-transferase product was bound by concanavalin A (Con A), retarded by *Vicia villosa* agglutinin, and retarded by RCA_{II}. This pattern of enzyme sensitivity and lectin interaction is the same as that seen with authentic oligosaccharides containing a single terminal β1,4-linked GalNAc (*6, 7, 14, 16, 17*). Oligosaccharides labeled with [^{3}H]Gal were analyzed in an analogous manner and found to have the properties of a dibranched complex type oligosaccharide with a single terminal β1,4-linked Gal present almost exclusively on the branch arising from the α1,3-linked core Man residue (*6, 7, 17*).

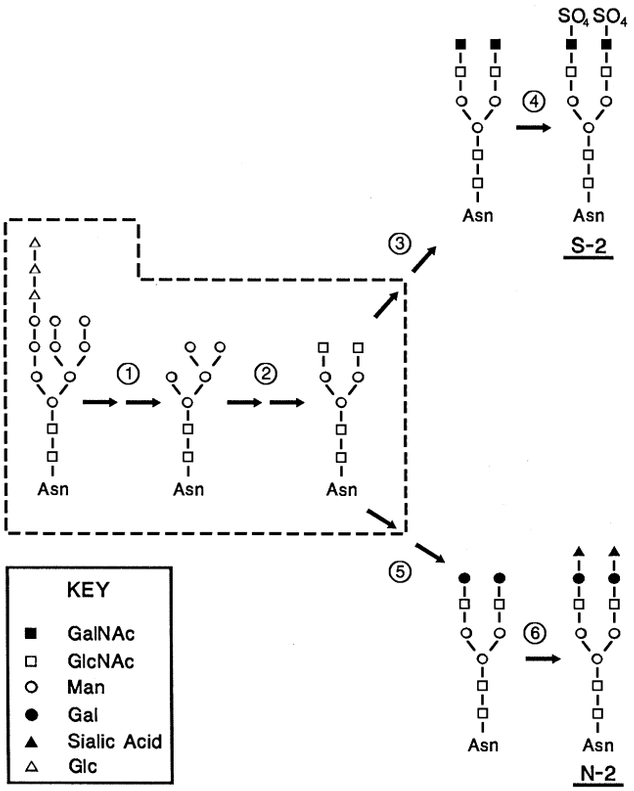

Fig. 1. Pathway for biosynthesis of sulfated and sialylated Asn-linked oligosaccharides present on pituitary glycoprotein hormones. Those portions of the pathway enclosed by the broken line are common for the synthesis of both sulfated and sialylated oligosaccharides. Steps leading to individual structures are indicated by the circled numbers.

Urinary hCG does not contain either sulfate or GalNAc on its Asn-linked oligosaccharides. The ability of hCGα to act as a substrate for the pituitary GalNAc-transferase suggested that GalNAc-transferase is not present in placenta, the major source of urinary hCG. Human placental membranes, prepared in the same fashion as pituitary membranes, did not transfer GalNAc to hCGα, whereas they did transfer Gal. We have also determined that a sulfotransferase with specificity for the sequence GalNAcβ1→4GlcNAcβ1→2Manα can be detected in pituitary but not placental membranes (*8, 18*). The lack of sulfate and GalNAc on the Asn-linked oligosaccharides of hCG, therefore, reflects the absence of both the sulfotransferase and the GalNAc-transferase in placenta and indicates that these are highly specialized enzyme activities that are not expressed in all cells or tissues.

These results indicate that a marker on the α-subunit of glycoprotein hormones is recognized by a GalNAc-transferase present in the pituitary. Since the oligosaccharides present on each of the substrates examined were identical, a feature of the α-subunit peptide must be recognized. Recognition of the hormone α-subunit represents the second example of protein-specific recognition by a glycosyl-transferase. Lysosomal enzymes contain an undefined feature encoded within their peptide, which is recognized by a GlcNAc-phosphotransferase and results in selective addition of GlcNAc-PO_4 to their Asn-linked oligosaccharides (*19, 20*). The apparent K_m for addition of GlcNAc-PO_4 to lysosomal enzymes (6 to 20 μ*M*) is similar

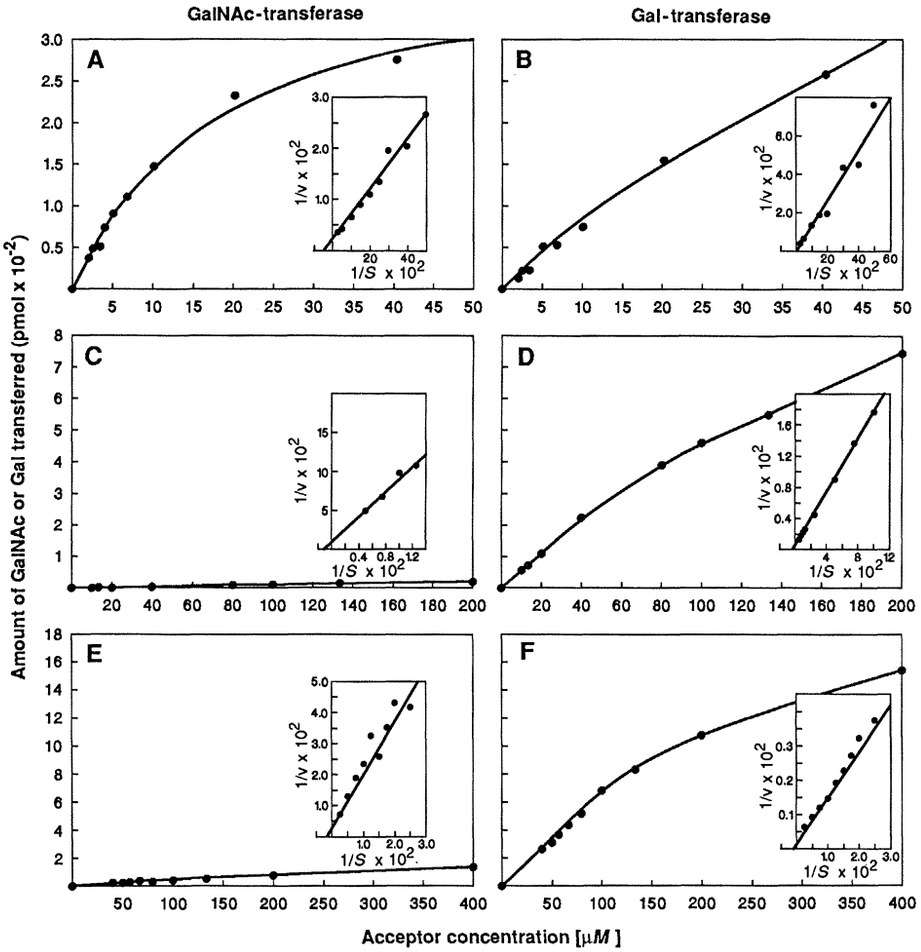

Fig. 2. Comparison of bovine pituitary GalNAc- and Gal-transferase activities. Sialic acid and Gal were released from Asn-linked oligosaccharides on hCGα, human transferrin, and a glycopeptide isolated from bovine IgG by digestion with clostridial neuraminidase and diplococcal β-galactosidase to produce oligosaccharides with structures identical to the $GlcNAc_2Man_3GlcNAc_2$ product of step 2 in Fig. 1. hCGα (**A** and **B**), human transferrin (**C** and **D**), and the glycopeptide (**E** and **F**), were then used as substrates for bovine pituitary GalNAc-transferase (A, C, and E) and Gal-transferase (B, D, and F). A total membrane fraction was prepared from frozen bovine pituitaries by homogenization in 30 m*M* tris, 120 m*M* KCl, and 5 m*M* magnesium acetate, *p*H 7.5, and sedimentation (125,000*g* for 4 hours) onto a cushion of 1.3*M* sucrose. Material at the interface was collected, diluted with the homogenization buffer, sedimented at 125,000*g* for 1.5 hours, and resuspended in 33 m*M* KCl and 17 m*M* magnesium acetate at 20 mg/ml and stored at −80°C. Each assay contained: (i) acceptors at the concentrations indicated, (ii) membranes, (iii) 0.8 m*M* [6-^{3}H]UDP-GalNAc (60 dpm/pmol) or 0.2 m*M* [6-^{3}H]UDP-Gal (220 dpm/pmol), and (iv) 25 m*M* Hepes, 1% Triton X-100, 10 m*M* adenosine triphosphate, 15 m*M* $MnCl_2$, 5.75 mTIU (milli–trypsin inhibitor units) of aprotinin, 1 μg of leupeptin, 1 μg of antipain, 1 μg of pepstatin, and 1 μg of chymostatin at *p*H 7.5 in a final volume of 50 μl. Assays were incubated for 90 min at 37°C and terminated by the addition of 450 μl of 1% SDS and 50 m*M* 2-mercaptoethanol followed by boiling for 10 min. Labeled acceptors were precipitated with 1% phosphotungstic acid/0.5*N* HCl, digested with pronase, and passed over a 1.5-ml column of Con A–Sepharose equilibrated in 10 m*M* tris, 0.15*M* NaCl, 1 m*M* $CaCl_2$, 1 m*M* $MnCl_2$, 0.02% azide and bovine serum albumin (1 mg/ml). Glycopeptides bearing dibranched oligosaccharides labeled with [^{3}H]GalNAc or [^{3}H]Gal were eluted with 500 m*M* α-methyl-mannoside, and their ^{3}H content was determined by scintillation counting. Lineweaver-Burk plots are shown as insets.

to the apparent K_m for addition of GalNAc to hCGα (25 μ*M*). These are among the lowest apparent K_m values thus far reported for glycosyltransferases.

The GalNAc-transferase can be viewed as having two recognition sites: one that interacts with the oligosaccharide and a second that interacts with the α-subunit peptide. When the peptide recognition marker is absent, the apparent K_m for GalNAc addition is similar to that observed for Gal addition by the Gal-transferase. The presence of the peptide recognition marker markedly reduces the apparent K_m for GalNAc addition.

Further studies are required to determine the identity of the recognition marker on the α-subunit and whether the same or a similar marker resides on the β-subunits of sulfated pituitary hormones such as LH and TSH. Associated β-subunits, however, appear to influence access to the recognition marker on α-subunits. Thus, LH has a greater proportion of sulfated oligosaccharides than FSH in three different animal species, even though LH and FSH are synthesized within the same pituitary cell, share a common α-subunit, and have β-subunits with highly homologous sequences (*1*, *7*). This suggests that FSHβ does not have a recognition marker for the GalNAc-transferase and that it limits access to the recognition marker on the associated α-subunit.

The presence of structurally unique Asn-linked oligosaccharides containing GalNAc and sulfate on LH in conjunction with their relative absence on FSH suggests that these oligosaccharides serve an important function for the pituitary glycoprotein hormones. The Asn-linked oligosaccharides may direct LH and FSH to separate granules within the gonadotroph or may modulate the biological activity of the secreted hormones, or both.

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Blocking of EGF-Dependent Cell Proliferation by EGF Receptor Kinase Inhibitors

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A systematic series of low molecular weight protein tyrosine kinase inhibitors were synthesized; they had progressively increasing affinity over a 2500-fold range toward the substrate site of epidermal growth factor (EGF) receptor kinase domain. These compounds inhibited EGF receptor kinase activity up to three orders of magnitude more than they inhibited insulin receptor kinase, and they also effectively inhibited the EGF-dependent autophosphorylation of the receptor. The most potent compounds effectively inhibited the EGF-dependent proliferation of A431/clone 15 cells with little or no effect on the EGF-independent proliferation of these cells. The potential use of tyrosine protein kinase inhibitors as antiproliferative agents is demonstrated.

MANY ONCOGENE PRODUCTS EXhibit protein tyrosine kinase (PTK) activity that is essential for their biological function (*1*). Similarly, early events in mitogenesis induction by growth factors are the ligand-induced autophosphorylation of the receptor and the phosphorylation of a host of intracellular substrates (*1, 2*). Insulin-induced autophosphorylation of its receptor, as well as hormone-induced tyrosine phosphorylation of specific intracellular target proteins, are the earliest identifiable biochemical events triggered by the hormone (*3*). The nullification of the PTK activity of insulin receptor (*3*) and of EGF receptor (*4*) by site-directed mutagenesis resulted in the elimination of their biological activity. Furthermore, low molecular weight PTK inhibitors that blocked insulin-dependent PTK activity of insulin receptor also inhibited insulin-induced lipogenesis and its antilipolytic effect in rat adipocytes (*5*). The involvement of PTK activity of many oncogene products in the expression of the transformed phenotype identifies these proteins as potential targets for selective chemotherapy and thus, for rational drug design. A series of low molecular weight compounds that inhibited EGF receptor PTK activity at concentrations that were a factor of 100 to 700 less than the concentrations needed to inhibit the PTK activity of insulin receptor were synthesized. The most potent EGF receptor kinase (EGFRK) blockers inhibited the EGF-dependent proliferation of A431/clone 15 cells.

Umezawa and colleagues (*6*) identified in the medium of an actinomycete a compound they termed erbstatin (Fig. 1), which inhibits the autophosphorylation of the EGF receptor in membranes of A431 epidermoid carcinoma cells. Low concentrations of erbstatin were required to inhibit the EGF receptor autophosphorylation (inhibition concentration $IC_{50} < 2.5$ μg/ml; 14 μ*M*), whereas its potency in inhibiting cyclic adenosine 3′,5′-monophosphate (cAMP)–dependent protein kinase was rather weak ($IC_{50} = 100$ μg/ml; 558 μ*M*).

We designed PTK inhibitors whose structures were initially patterned after erbstatin. Three criteria that guided us were as follows. (i) The compounds should be competitive with the substrate of EGFRK and not with adenosine triphosphate (ATP). The PTK inhibitors quercetin (*7*) and genistein (*8*), which compete with ATP, inhibit other protein kinases and are highly cytotoxic. (ii) As a test of selectivity we chose compounds that inhibited EGFRK much better than the insulin receptor kinase (IRK), which is structurally and functionally highly homologous to EGFRK in its kinase domain (*9*). (iii) The compounds should be soluble both in water and in mildly hydrophobic solvents such as alcohols and dimethyl sulfoxide. These solubility properties enhance the probability that such compounds will be

Table 1. Potency of EGFRK inhibitors. The inhibition constants quoted in the table were determined from Dixon plots with three substrate concentrations for each inhibitor, as described in legend to Fig. 2. Together with Fig. 2 the benzylydene compounds represent a series in which their affinity toward EGFRK is increased 2940-fold (2500/0.85) through specific substitutions.

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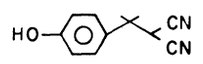
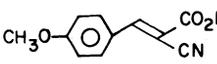
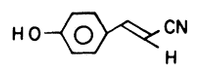
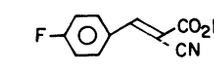
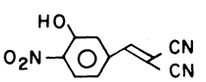
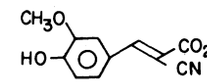
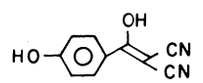
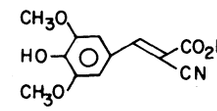
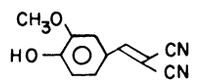
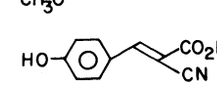
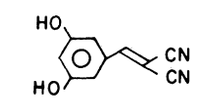
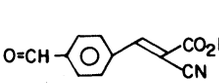
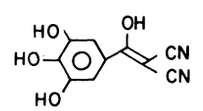
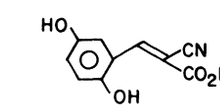
13		2500	20		833
14		1333	21		833
15		150	22		267
16		77	23		233
17		67	24		166
18		10	25		47
19		3.3	26		24

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