Differentiation of Coelenteramide Sulfates from Phosphates by Hydrogen/Deuterium Exchange Coupled with Ion Trap Mass Spectrometry

Issei DOI,1 Masaki KUSE,2 Yosuke NAKASHIMA,1 Naoki TANI,1 and Minoru ISobe3*

1 Laboratory of Organic Chemistry, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya, JAPAN
2 Chemical Instrument Division, Research Center for Materials Science, Nagoya University, Nagoya, JAPAN
3 Institute for Advanced Research, Nagoya University, Nagoya, JAPAN

In general, it is difficult to distinguish between sulfate and phosphate groups in natural products, because these groups are found only in trace amounts in natural products. We have devised a hydrogen/deuterium (H/D) exchange method using the liquid chromatography mass spectrometry technique for establishing a general methodology to distinguish between sulfated and phosphorylated compounds. Sulfate- or phosphate-containing compounds were differentiated by the H/D exchange method based on the number of exchangeable hydrogen in deuterated sulfate (−SO3D) and phosphate (−PO3D2), respectively. In this paper, we describe an application of this method to a natural product and in particular to Watasenia oxyluciferin that is found in the firefly squids (Hotaru-ika in Japanese) for the purpose of identifying coelenteramide disulfate.

(Received November 18, 2008; Accepted December 24, 2008)

1. Introduction

Natural products containing either sulfate or phosphate group are rarely found in nature.1,2 For example, during the formation of cuticle in insects, phenol residue of tyrosine usually converts to sulfate, phosphate, or β-glycoside increasing its solubility in water.3-6 Then, the resultant polar compounds are transferred to cuticle-producing cells. Depending upon the evolutionary state of insects, these polar substituents have varying tyrosine transportation mechanisms which differ according to the species of insects. Periplaneta and Drosophila use tyrosine sulfate and tyrosine phosphate, respectively, for cuticle formation. However, it is difficult to distinguish between sulfate and phosphate groups, particularly when the given samples are isolated in trace amounts. The usual method used to distinguish between sulfate and phosphate groups using the high-resolution mass spectrometry (MS) technique requires a 0.009 u difference. Phytosulfokine, a plant hormone used for proliferating dispersed plant cells in the culture, was isolated from a single mesophyll cell and was determined as a sulfated compound using fast atom bombardment mass spectrometry (FAB-MS) by Sakagami et al. in 1996.7

Sulfated and phosphorylated compounds can be easily identified on the basis of their fragmentation patterns, because only phosphorylated compounds often show a −18 u fragmentation. However, this could be misleading for sulfated compounds such as sugars that have multiple hydroxyl groups.8 On the other hand, a sulfatase enzyme may be useful to confirm the presence of sulfate ions, but we have reported earlier that sulfatase also hydrolyzes phosphates, such as daidzein 7-O-phosphate and genistein 7-O-phosphate, because this was determined by carbon nuclear magnetic resonance (NMR) and phosphorus NMR experiments.9 However, NMR analysis is a useful method only when a compound is available in milligrams.

We have used liquid chromatography mass spectrometry (LC-MS) for conducting the hydrogen/deuterium (H/D) exchange, and then we have directly analyzed these compounds available in trace amounts using an electrospray ionization quadrupole time-of-flight mass spectrometry (ESI-Q-TOF-MS). This is a very beneficial method to compare the original LC-MS data in water (H2O) + acetonitrile (CH3CN) solvent system with a similar LC-MS data run in a deuterium oxide (D2O) + acetonitrile (CH3CN) solvent system. We found this method to be applicable to an unstable compound found in a luminous substance obtained from photogenic organs.

All the hydrogen atoms of the hydroxyl or amino groups exchange rapidly with deuterium in a D2O solvent. In this study, we used ion trap mass spectrometry (IT-MS) and tandem mass spectrometry (MS/MS). IT-MS machine was operated alternately between positive and negative ion modes while scanning.10-14 A sulfate group possesses a single exchangeable hy-
hydrogen which is present in the hydroxyl group of sulfate, whereas a phosphate group has two exchangeable hydrogens which are present in the two hydroxyl groups of phosphate. The number of exchangeable hydrogens always show a 1 u difference between sulfate and phosphate groups irrespective of the ion mode (Fig. 1). 1 u difference makes it very easy to distinguish deuterated sulfate from deuterated phosphate by MS. In Fig. 1, all the structures in protonic solvents exhibit the same nominal mass numbers for sulfate and phosphate groups under acidic and basic conditions. In contrast, these values are different in deuterated solvents, such as D2O, methyl alcohol-OD. Moreover, sulfate groups always exhibit a nominal mass which is one unit smaller than that of phosphate groups under identical conditions. This phenomenon was previously utilized to determine two isoflavones which were phosphorylated in original ESI- and FAB-MS method.15) However, this method was not applicable to subsequent studies dealing with unstable chromophores originating from photogenic organs of the squid Watasenia scintillans.

Goto et al. found that luciferin and oxyluciferin of the firefly squid (Watasenia scintillans) were coelenterazine disulfate (1)16, 17) and coelenteramide disulfate (2),18) respectively (Fig. 2). Tsuji et al. recently reported that the luminescence mechanism of this squid requires molecular oxygen, adenosine triphosphate (ATP), and magnesium ion for the luciferase to emit light.19) Considering these authentic samples, we applied the H/D exchange MS method for elucidating the structure of Watasenia luciferin, which we extracted from homogenates (Compound X) of 50 pieces of the arm photogenic organs.

2. Experimental

2.1 Chemicals

Hypergrade acetonitrile (for LC-MS) and trifluoroacetic acid-D (CF3COOD, D; 99.8%), trifluoroacetic acid, and deuterium oxide (D2O; 99.9%, 25 g) were purchased from Merck (Germany), Nacalai tesque (GR, Japan), and Cambridge Isotope Laboratory, respectively. The water was used Milli-Q grade. The deuterium solvent was freshly opened just before applying gradient conditioning (D2O/acetonitrile 0.025) for HPLC analysis at room temperatures. The solvent could be used for a day when capped under an argon balloon.

2.2 Instrumentation

Luminescent activity was measured using a luminescence spectrometer, AB-2200-R (ATTO, Tokyo, Japan). Proton NMR spectra were recorded on a JEOL A-600 (JEOL, Tokyo, Japan) at 600 MHz. Chemical shifts (δ) were reported in parts per million relative to tetramethylsilane (δ 0.00), D2O (δ 4.65), or dimethylsulfoxide-d6 (δ 2.49) that were used as an internal standard. Coupling constants (J) are given in Hz. Carbon NMR spectra
were recorded on a JEOL A-600 at 150.9 MHz. Chemical shifts (δ) are reported in parts per million relative to dimethylsulfoxide-d₆ (δ 45.0) that was used as the internal standard. Coupling constants (J) are reported in Hz. Low-resolution and high-resolution FAB-MS were measured using a JEOL JMS-700 (JEOL, Tokyo, Japan). Fluorescence spectra were measured using a fluorescence spectrometer, FP-920 (JASCO Co., Ltd., Tokyo, Japan) that was coupled with an appropriately adjusted high performance liquid chromatography (HPLC) system (JASCO Co., Ltd., Tokyo, Japan) employing Develosil ODS-HG-5 (Nomura Co., Ltd., Aichi, Japan, 250 × 4.6 mm i.d.) columns. For analysis, columns were equilibrated with a H₂O-CH₃CN (3:1) mobile phase containing 0.025% trifluoroacetic acid at a 0.5 mL/min flow rate. The column effluent was monitored using the 350 nm (λex) ultraviolet (UV) light as the excitation wavelength and fluorescence (FL) was measured at 530 nm (λem).

2.3 Samples

2.3.1 Coelenterazine diphosphate (3)

Coelenterazine (20 mg, 0.048 mmol), which was prepared according to Goto’s method, was added to a mixture of tetrahydrofuran (1.0 mL) and pyridine (7.3 mmol, 0.58 mL). Phosphoryl chloride (POCl₃, 2.6 mmol, 0.24 mL) was added dropwise to this resultant solution and was simultaneously stirred under an argon atmosphere at 0°C. Then, it was allowed to stand at room temperature for 40 min. After cooling, water (33 mmol, 0.58 mL) was added to the reaction mixture and the produced yellow solid was filtered and washed with acetonitrile containing 0.025% trifluoroacetic acid to yield 21 mg (77% yield) of coelenterazine diphosphate 3 which is a brown-yellow solid having a mp of 101–104°C (decomposition). Coelenterazine diphosphate was very unstable. UV (methanol) λmax (log ε) 417 (0.34), 328.5 (0.38), 257.5 (1.17). FL (H₂O/CH₃CN; 3:1) λem 520 nm. Proton NMR (dimethylsulfoxide-d₆, 600 MHz), δ 4.05 (2H, s), δ 4.33 (2H, s), δ 7.0–7.8 (m) ppm. Carbon NMR (dimethylsulfoxide-d₆, 150 MHz), δ 27.3, 28.4, 108.2, 119.9 (d, J = 29 Hz), 120.2 (d, J = 51 Hz), 126.6, 127.3, 128.3, 129.0, 129.5, 134.1, 137.7, 139.2, 147.3, 149.9, 152.1 ppm. Phosphorus NMR (dimethylsulfoxide-d₆, 150 MHz), δ −5.54 (d, J = 15 Hz), −4.89, −4.68 ppm. FAB-MS (m-nitrobenzyl alcohol) m/z 582 [M − H]⁻. High resolution MS (FAB/m-nitrobenzyl alcohol) calculated for C₃₀H₂₂O₉N₃P₂ 570.0831, found 570.0845 [M − H]⁻.

2.4 Extract from Watasenia arm photogenic organs (Compound X)

2.5 Measurement of Watasenia bioluminescence activities using coelenterazine disulfate and diphosphate

The arm photogenic organs of Watasenia scintillans (wet 225 mg, ca. 50 specimens) were homogenized in a 70% methanol—water (3 mL at 0°C) medium. The resultant suspension was filtered through a polytetrafluoroethylene membrane and evaporated to remove methanol. The residue was purified by HPLC [ODS-UG-5 column (250 × 4.6 mm), and a guard column (ODS-5, 10 × 4.0 mm) using 25% acetonitrile + water containing 0.025% trifluoroacetic acid as the mobile phase. The fraction exhibiting fluorescence was collected and concentrated to yield 50 μg of a blue fluorescent compound X.

2.6 Liquid chromatography electrospray ionization ion trap tandem mass spectrometry (LC-ESI-IT-MS/MS)

The entire LC-MS and MS/MS experiments were conducted using a house-assembled HPLC system (JASCO Co., Ltd., Tokyo, Japan) employing Develosil ODS-HG-5 (Nomura Co., Ltd., Aichi, Japan, 150 × 3.0 mm i.d.) columns, and measurements were conducted using an ion trap High Capacity Trap (HCT) Plus mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an orthogonal ESI source. Columns were equilibrated using 260 μL of water containing 0.025% trifluoroacetic acid at a 10 μL/min flow rate. The columns were subsequently developed using a linear gradient ranging from 0% to 100% of acetonitrile containing 0.025% trifluoroacetic acid for 40 min at a 5 μL/min flow rate with the Pre-Packed-Gradient program. The resulting eluents were directly injected into sample inlet, which has a non-split system, of the mass spectrometer. We performed a complete MS analysis at femto-mol/mL concentrations without causing any loss of precious natural products. For conducting hydrogen/deuterium ex-
change experiments, D₂O containing 0.025% CF₃COOD was prepared quickly and degassed at room temperature within 10 min to avoid the exchange of deuterium with hydrogen in air. Then, it was equilibrated with D₂O (280 µL) containing 0.025% CF₃COOD at a 10 µL/min flow rate. It was developed with a linear gradient ranging from 0 to 100% acetonitrile containing 0.025% CF₃COOD at a 5 µL/min flow rate. The column effluent was monitored using a 350 nm ultraviolet (UV) light (λex) as the excitation wavelength, and the fluorescence (FL) was measured at 530 nm (λem). Thereafter, the column effluent was introduced into an electrospray nebulizer without splitting. All the samples for MS and MS/MS were diluted with water containing 0.025% trifluoroacetic acid to produce 2 mM samples at room temperature, with hydrogen in air. Then, the column was equilibrated with hydrogen in air. The column effluent was monitored with hydrogen in air. Thereafter, it was monitored with hydrogen in air.

### Results and Discussion

#### 3.1 H/D exchange experiment combined with LC-ESI-IT-MS performed on coelenterazide disulfate (1) and diphosphate (3)

Coelenterazide disulfate 1 and diphosphate 3 were subjected to our H/D exchange analysis conditions, and the number of exchangeable hydrogens were measured by LC-ESI-IT-MS, enabling us to simultaneously obtain fluorescence and UV spectra from trace amounts of compounds. We expected three exchangeable hydrogens for 1, which were present in the secondary amino group of imidazopyrazinone and two hydroxyl groups of disulfate. In a similar way, we expected five exchangeable hydrogens for 3, which were present in the secondary amino group of imidazopyrazinone and four hydroxyl groups of diphosphate. Deprotonated molecular peaks corresponding to 1 and 3 were observed at m/z 582.0 and m/z 582.2 [M−H]⁻ in the negative ion mode. No peaks were observed in the positive ion mode (Fig. 3A, C).

In the mass spectrum shown in Fig. 3, deprotonated molecular peaks of 1 mainly appeared at m/z 290.4 [M−2H]^2⁻ as a double-charged ion, and weak ion peaks at m/z 582.0 [M−H]⁻ represented the single-charged ions. On the other hand, the deprotonated molecular peak of 3 appeared at m/z 582.2 [M−H]⁻ representing a single-charged ion with high intensity. After deuteration, both compounds were analyzed under identical conditions (Figs. 3B, D). Deprotonated molecular peaks of 1 were shifted to m/z 584.0 [M−D]⁻ and m/z 290.9 [M−2D]^2⁻ from m/z 582.0 [M−H]⁻ and m/z 290.4 [M−2H]^2⁻, respectively, whereas the deprotonated molecular peaks of 3 were shifted to m/z 586.3 [M−D]⁻ and m/z 292.0 [M−2D]^2⁻ from m/z 582.2 [M−H]⁻ and m/z 290.5 [M−2H]^2⁻, respectively. Based on the detected molecular peaks, the calculated number of exchangeable hydrogens on 1 was three, while that of 3 was five. These correspond to our expected numbers. Throughout the analyses, we found that it is easy to distinguish between 1 and 3 based on the number of exchangeable hydrogens and the relative peak intensities of the single-charged ions in the negative ion mode.

#### 3.2 H/D exchange experiment combined with LC-ESI-IT-MS performed on coelenteramide disulfate (2) and diphosphate (4)

Coelenteramide disulfate 2 and diphosphate 4 were analyzed using LC-ESI-IT-MS/MS with the analysis condition same as that used for 1 and 3 (Fig. 4). We expected that the number of exchangeable hydrogens for 2 would be three, which were present in the secondary amino group of amidopyrazine and two hydroxyl

---

Fig. 3. Mass spectra of 1 and 3 before and after conducting the H/D exchange method in the negative ion mode: (A) H form of coelenterazide disulfate 1, (B) Coelenterazide disulfate 1 after H/D exchange, (C) H form of coelenterazine diphosphate 3, (D) Coelenterazine diphosphate 3 after H/D exchange. HPLC chromatograms of 3 and 1, and compound X were monitored by a fluorescent spectrometer.
groups of disulfate. In addition, we expected the number of exchangeable hydrogens for 4 to be five, which were present in the secondary amino group of imidazopyrazinone and four hydroxyl groups of diphosphate.

Protonated and deprotonated molecular peaks of these compounds were observed under both positive and negative ion modes. The deprotonated molecular peaks of 2 appeared at \( m/z \) 570.0, whereas that of 4 appeared at \( m/z \) 570.0 \([M-H]^-\) in the negative ion mode (Figs. 4A, C). When analyzed in the negative ion mode, 2 has two exchangeable hydrogens, whereas 4 has four exchangeable hydrogens. The deprotonated molecular peaks of 2 showed patterns similar to 1; the double-charged ions of 2 were mainly observed at \( m/z \) 284.5 \([M-2H]^2+\), and deprotonated molecular peaks of 4 appeared at \( m/z \) 570.0 \([M-H]^-\) with high intensity.

The retention time of 4 was same as that for sulfate 2 in the HPLC chromatogram (Fig. 4). Then, we analyzed 2 and 4 with MS/MS by selecting the precursor ion at \( m/z \) 570.0 \([M-H]^-\). Based on the number of exchangeable hydrogens, single-charged ions were selected as precursor ions for tandem mass spectra analysis, although the analysis could have afforded many fragment ions had double-charged ions been selected as precursor ions. Compounds 2 and 4 had fragment ion peaks at \( m/z \) 490.0 or \( m/z \) 490.1, respectively, corresponding to a loss of SO3 from 2 and PO3H from 4 (loss of 80 u). The fragment ion peak at \( m/z \) 552.0 was observed only for the tandem mass spectrum of 4, which was due to dehydration of 4 (no fragment ion peak at \( m/z \) 552.0 was observed in the disulfate). After deuteration, the deprotonated molecular peaks of 2 were shifted to \( m/z \) 572.0 \([M-D^-]^-\) and \( m/z \) 284.9.
from m/z 570.1 [M−H]− and m/z 284.5 [M−2H]2−, respectively. The deprotonated molecular peaks of 4 were shifted to m/z 574.1 [M−D]− and m/z 286.1 [M−2D]2− from m/z 570.1 [M−H]− and m/z 284.5 [M−2H]2−, respectively (Figs. 4 B, D). A successive tandem mass spectra analysis of the precursor ions at m/z 572.0 [M−D]− of 2 afforded fragment ion peaks at m/z 492.0, which correspond to a loss of SO3 (loss of 80 u) (Fig. 5). On the other hand, tandem mass spectra analysis of precursor ions at m/z 574.1 [M−D]− of 4 afforded fragment ion peaks at m/z 493.1 and m/z 554.0, which correspond to a respective loss of PO3D (loss of 81 u) and D2O (loss of 20 u). In the positive ion mode, the protonated molecular peaks of both 2 and 4 appeared at m/z 572.0 [M+H]+. The protonated molecular peaks of 2 shifted to m/z 576.0 [M+D]+ after deuteration. In contrast, the protonated molecular peaks of 4 shifted to m/z 578.2 [M+D]+. Based on these molecular peak assignments, the calculated number of exchangeable hydrogens on 2 was three, whereas that of 4 was five. These numbers of exchangeable hydrogens correspond to the expected ones in both positive and negative ion modes.

3.3 H/D exchange experiment combined with LC-ESI-IT-MS performed on an extract from Watasenia arm photogenic organs

This compound had the same fluorescent spectrum as 2 and 4; however, it was impossible to distinguish

Table 1. m/z Value of H and D Form of Coelenterate Sulfate, Coelenterate Phosphate, and Extracts from Watasenia Arm Photophores, Respectively

<table>
<thead>
<tr>
<th>Compound</th>
<th>[M−H]−</th>
<th>[M−D]−</th>
<th>[M−2H]2−</th>
<th>[M−2D]2−</th>
<th>[M+H]+</th>
<th>[M+D]+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coelenterazine disulfate 1</td>
<td>Calcd. m/z 582.1</td>
<td>584.1</td>
<td>290.5</td>
<td>291.0</td>
<td>584.1</td>
<td>586.1</td>
</tr>
<tr>
<td>Observed m/z 582.0</td>
<td>584.0</td>
<td>290.4</td>
<td>290.9</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>Coelenterazine diphosphate 2</td>
<td>Calcd. m/z 582.1</td>
<td>586.1</td>
<td>290.5</td>
<td>292.0</td>
<td>584.1</td>
<td>590.1</td>
</tr>
<tr>
<td>Observed m/z 582.2</td>
<td>586.3</td>
<td>290.5</td>
<td>292.0</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>Coelenteramide disulfate 3</td>
<td>Calcd. m/z 570.1</td>
<td>572.1</td>
<td>284.5</td>
<td>285.0</td>
<td>572.1</td>
<td>576.1</td>
</tr>
<tr>
<td>Observed m/z 570.0</td>
<td>572.0</td>
<td>284.5</td>
<td>284.9</td>
<td>572.0</td>
<td>576.0</td>
<td></td>
</tr>
<tr>
<td>Coelenteramide diphosphate 4</td>
<td>Calcd. m/z 570.1</td>
<td>574.1</td>
<td>284.5</td>
<td>286.0</td>
<td>572.1</td>
<td>578.1</td>
</tr>
<tr>
<td>Observed m/z 570.1</td>
<td>574.1</td>
<td>284.5</td>
<td>286.1</td>
<td>572.1</td>
<td>578.2</td>
<td></td>
</tr>
<tr>
<td>Compound X from Watasenia extracts</td>
<td>Observed m/z 570.0</td>
<td>572.0</td>
<td>284.4</td>
<td>285.0</td>
<td>572.1</td>
<td>576.0</td>
</tr>
</tbody>
</table>

Calcd.: calculated
between sulfate and phosphate groups based on the fluorescent spectra. Compound X obtained from the Watasenia extract was also analyzed under conditions which were identical to those applied for coelenteramide disulfate and diphasphate (Fig. 6).

Deprotonated molecular peaks of compound X appeared at m/z 570.0 [M − H]− as a single-charged ion and at m/z 284.4 [M − 2H]2− as a double-charged ion. Tandem mass spectra analysis of the precursor ion at m/z 570.0 [M − H]− afforded fragment ion peaks at m/z 490.1, which correspond to a loss of SO3 (loss of 80 u). After deuteration of compound X, the deprotonated molecular peaks were shifted to m/z 572.0 [M − D]− and m/z 285.0 [M − 2D]2− from m/z 570.0 [M − H]− and m/z 284.4 [M − 2H]2−, respectively (Fig. 6). Successive tandem mass spectra analysis of precursor ions at m/z 572.0 [M − D]− afforded fragment ion peaks at m/z 492.1, which corresponds to a loss of SO3 (loss of 80 u). In the positive ion mode, protonated molecular peaks of compound X appeared at m/z 572.1 [M + H]+ and after deuteration, these peaks shifted to m/z 576.0 [M + D]+. Based on these molecular peaks, the calculated number of exchangeable hydrogens on Compound X was three, while the MS/MS fragmentation patterns and ionization efficiency of single-charged ion peaks was similar to that of 2. On the basis of these findings, we have concluded that the compound X is definitely a disulfate 2.

3.4 Measurement of Watasenia bioluminescent activities using coelenterazine disulfate and diphasphate

We have investigated the luminescent activity for Watasenia scintillans using synthesized 3 that followed Tsuji’s protocol. Watasenia bioluminescent activity was measured at a trace level of activity using 3 or coelenterazine, whereas 1 affected a large amount of bioluminescent activity in the Watasenia luciferin–luciferase reaction (the ratio of relative light intensity for 1 min in Watasenia bioluminescence was 1 : 3 : coelenterazine = 100 : 8 : 6) (Fig. 7). From these results, we also confirmed that luciferin was coelenterazine disulfate 1.

4. Conclusion

Herein, we have extended our H/D exchange methodology to microanalyses of sulfated and phosphorylated compounds using LC-ESI-IT-MS. Synthetic 1 and 3 were analyzed by IT-MS. We found that the ionization efficiency of the double-charged ion peaks and their fragmentation patterns were very different for 1 and 3. For 2 and 4, the same relationship was observed. Watasenia bioluminescent activity also indicated that Watasenia luciferin was 1. By comparing with the data (Table 1) obtained on synthetic compounds, we assigned the structure of Compound X to 2 with certainty. Based on these analyses, we have proved that our H/D exchange method is applicable to distinguish a small amount of a natural product having a sulfate group and/or phosphate group using an IT-MS coupled with a HPLC.

Acknowledgement

We acknowledge the financial support of the Grant-in-Aid for Specially Promoted Research [16002007 (2004)] from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan. Authors are grateful for being the recipients of a JSPS Grant-in-Aid for Encouragement of Young Scientists [19780087 (2007)] to M. Kuse as well as to the 21st Century Center of Excellence (COE) program for scholarship to N. Tani and I. Doi, and for the global COE program, and Ono Pharmaceuticals Co., Ltd. for financial support to I. Doi. The authors gratefully acknowledge Dr. M. O. Sydnes’ proofreading of this manuscript.

References


Keywords: Ion trap mass spectrometry, Hydrogen/deuterium exchange method, Coelenterate, Disulfate, Diphasphate